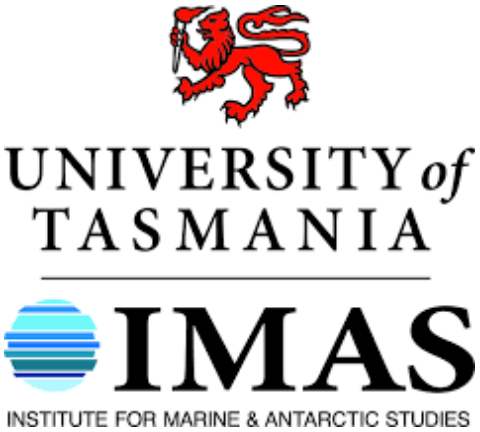

Isolation and Selection of Probiotic Candidates from Gastrointestinal Tracts of Teleosts and Molluscs

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Declaration of Originality

I declare that all the materials presented in this thesis is based on original research and contain no material previously published either in print or electronically, except where due to references is made in the text. None of this material has been used by me or any other person to attain another tertiary, degree or award, and this thesis has not been submitted to any other tertiary institution.

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Abstract

Abalone aquaculture is increasingly important because of the progressive decline of wild catch abalone overtime, yet the world's demand for the seafood is continuously increasing. However, slow growth rates and disease are two major constraints in the development of abalone aquaculture. One way to deal with these issues is the use of beneficial bacteria, generally known as probionts. This approach has been confirmed to enhance the growth and disease resistance of many cultured terrestrial and aquatic animals. However, viability is still regarded as a major challenge when probionts are applied in aquaculture animals. This is probably because the probiotic strains are isolated from terrestrial organisms, which in fact have very different environmental conditions compared to aquatic species. Given these issues, it has been suggested that probionts for aquaculture species should be isolated from aquatic animals. Therefore, this study aimed at isolation of bacteria associated with gastrointestinal tracts (GITs) of aquatic teleosts and molluscs, and screening for probiotic candidates. The GIT was selected as a source of bacterial isolation, because GITs are important sites where digestion and absorption of feed occurs, as well as one of the most common entry ports for bacterial infections.

The study was started by isolating 230 endogenous bacteria from GITs of 155 aquatic teleosts and molluscs collected from different aquatic environments (recirculating aquaculture systems, flow-through aquaculture systems and wild environments). Out of the 230 intestinal bacteria, 24 isolates displayed a capacity to synthesize digestive enzymes, either protease, alginate lyase or cellulase. The other 22 isolates produced antimicrobial compounds against at least one of eight bacterial pathogens (*Vibrio alginolyticus, Vibrio parahaemolyticus, Vibrio harveyi, Vibrio anguillarum, Vibrio proteolyticus, Yersinia ruckeri, Aeromonas hydrophila,* and *Listeria monocytogenes*). Then, five bacteria having either the highest enzymatic or the broadest antimicrobial activity were selected for studying

their viability in simulated GITs' environments. The results showed that the five bacteria displayed good viability in the simulated GITs of abalone, being tolerant to a low pH, to gastric enzymes (trypsin and pepsin) and to surfactants like bile-salt in a simulated intestinal juice. These results indicate that the five bacteria have a good viability and potential capacity to colonize the GITs of abalone.

To confirm these *in vitro* results in live animals, three bacteria were selected to be used as feed supplement in abalone in a mixture of 2 bacteria (*Bacillus amyloliquefaciens* subsp. *plantarum* and *Enterobacter ludwigii*; 2P) or three bacteria (*B. amyloliquefaciens* subsp. *plantarum*, *E. ludwigii* and *Pediococcus acidilactici*; 3P). These bacteria were incorporated onto *Gracillaria* sp., which is a natural feed of abalone and was fed to juvenile abalone, *Haliotis asinina*, for 62 days. The results showed that abalone fed on 2P or 3P-supplemented diets had significantly higher growth rates (both wet weight and shell length) compared to the abalone receiving unsupplemented diets (control). Abalone receiving the 2P or 3P supplemented diet showed similar survival to the abalone in the control, which was >72 %. These results suggest that it is feasible to use a mixture of *B. amyloliquefaciens* subsp. *plantarum*, and *E. ludwigii* to increase production yield of abalone, *H. asinina*. However, further studies such as the use of different probionts and cell concentrations are required to enhance survival rate of abalone and appropriately commercialise the findings.

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These are conferences and publications from this thesis:

Conferences:

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2. Amin, M., Adams, M., Bolch, C.J.S., Burke, C.M., 2016. *In vitro* screening of lactic acid bacteria isolated from gastrointestinal tract of Atlantic Salmon (*Salmo salar*) as probiont candidates. Aquaculture International. 25, 485-498.

List of Abbreviations

BLIS – Bacteriocin-Like Inhibitory Substances

CFS – Cell-Free Supernatant

CFSn – Neutralized Cell-Free Supernatant

FTAS – Flow-Through Aquaculture System

GITs – Gastrointestinal Tracts

LAB – Lactic Acid Bacteria

RAS – Recirculating Aquaculture System

SIJ – Simulated intestinal juice

SSJ – Simulated stomach juice

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Chapter 1 : General Introduction

1.1. Abalone: species diversity and markets

Abalone is an economically important marine gastropod from the family of *Haliotidae*. Seventy-five to 100 species of abalone have been identified worldwide. The majority of these species inhabit temperate regions, while small numbers populate tropical regions. Of these, more than 20 species have been classified as commercially-important species (Jarayabhand and Paphavasit, 1996). Among which are: *Haliotis squamata, Haliotis gigantea* in Japan (Iehata et al., 2014), *Haliotis discus hannai, Haliotis diversicolor* in Korea (Park and Kim, 2013b) and China (Zhao et al., 2012), *Haliotis rufescens* in USA and Chile (Valenzuela-Munoz and Gallardo-Escarate, 2013), *Haliotis iris* in New Zealand (Hadi et al., 2014), *Haliotis midae* in South Africa (Erasmus et al., 1997), *Haliotis laevigata*, *Haliotis rubra* in Australia (Harris et al., 2005), and *Haliotis asinina* L in the Philippine and other Southeast Asian Countries (Capinpin and Corre, 1996).

Abalone is known as "the emperor of the seashells" or "ginseng in the ocean", due to its high nutritional contents and health-promoting properties for humans (Mateos et al., 2010; De Zoysa, 2013). As a result, demands for abalone are continuously increasing from various countries such as Taiwan, Japan, USA, and Canada (Cook, 2014). Previously, the demand was mainly supplied from abalone fisheries. However, since 1990s, the total annual catch has gradually declined (Cook and Gordon, 2010). Several factors which contributed to the decline are: overexploitation, illegal fisheries, infectious disease, and habitat degradation. Recent data reported that the total quantity of abalone obtained from fisheries dropped sharply, from 20,000 metric tons (mt) in the 1970s to only 6,500 mt in 2015 (Cook, 2016). As a consequence, the supply of market-size abalone was insufficient. Thus, efforts to develop an artificial culture of the marine shellfish in several countries have been increased.

1.2. Abalone aquaculture

Up to now, abalone aquaculture has been well-established in several countries such as Chile (Flores-Aguilar et al., 2007), USA (Valenzuela-Munoz and Gallardo-Escarate, 2013), Australia (Gilroy and Edwards, 1998), China (Zhao et al., 2012), Korea (Park and Kim, 2013b), South Africa (Britz et al., 1997), Japan (Sawabe et al., 1998) and the Philippines (Capinpin et al., 1999). The total quantity of abalone harvested from farms has begun to significantly contribute to the international abalone markets (Gordon and Cook, 2013). Cook (2016) reported that total abalone production on farms increased from only 50 mt in the 1970s to 129,287 mt in 2015. Previously, the country which produced the highest quantity was Japan, followed by Australia, New Zealand, the USA, Mexico and South Africa consecutively (Jarayabhand and Paphavasit, 1996). However, current data indicated that the largest abalone-producing country is China, sharing 88% of total world's abalone production in 2015, followed by South Korea with 7% of share. Meanwhile, other countries such as South Africa, Chile, Japan, USA, and Australia contributed less than 3 % of the total production (Cook, 2016).

Abalone aquaculture begins by producing larvae in hatcheries. Many abalone-exporting countries currently have already produced their own brood stock such Australia (Kube et al., 2007), China (Zhang et al., 2004), United states (Salinas-Flores et al., 2005)and Korea (Park and Kim, 2013a). During larval stages, abalone fed with benthic diatoms (Kawamura and Takami, 1995; Gallardo and Buen, 2003; Uriarte et al., 2006; Capinpin Jr, 2008; Matsumoto et al., 2015; Sanchez-Saavedra and Nunez-Zarco, 2015). After reaching juvenile stages (10-30 mm), they are moved into grow-out aquaculture systems, either land-based farms (e.g., tanks) or sea-based farms such as sea cages (Flores-Aguilar et al., 2007). Both culture systems are generally designed to promote even distribution of all animals, ready access to feed, minimum contact among animals and feed with fecal wastes, good

water flow and exchange, and minimum human disturbance. During the grow-out process, abalone are fed with either macroalgae (Kawamura and Takami, 1995), kelps (Flores-Aguilar et al., 2007) or manufactured pellets. A study by Cook (2016) demonstrated that abalone cultured in sea-based farms and fed with their natural diets such as macroalgae or kelps could reduce significantly operational cost. This is one of the strategies which have been developed in China and which make China the largest abalone producer nowadays. However, there are still several challenges which have continued to delay the development of abalone aquaculture.

1.3. Constraints in abalone aquaculture

The slow growth rates and the outbreak of infectious diseases are faced as two major constraints in abalone aquaculture industries (Burr et al., 2005; Uriarte et al., 2006; Silva-Aciares et al., 2011; Bidhan et al., 2014; Huddy and Coyne, 2015). Many studies presented that abalone species grew relatively slow compared to other aquatic species. To reach a marketable size of 80 -100 mm, several abalone species required 2-5 years, 5 years for *H. midae* (Huddy and Coyne, 2015), 4-5 years for *H. iris* (Hadi et al., 2014), 4 years for *H. Laevigata* (Dunstan et al., 2007), and 2-5 years for *H. asinina*. (Hahn, 1989). The slow growth causes longer culture periods and leads to more operational costs. Previous studies suggested that the slow growth was frequently associated with the low quality and digestibility of diets. Natural feed of abalone such as red and green macroalgae (Angell et al., 2012) are considered to have low digestibility, indicated by the high value of feed conversion ratio (FCR) (7 – 10) (Bautista-Teruel and Millamena, 1999; Mulvaney et al., 2013).

Furthermore, abalone lack an adaptive immune system, therefore being considered to be very vulnerable to infectious diseases (Jiang et al., 2013a). Like other invertebrate

animals, abalone rely only on their innate immune systems against infectious pathogens. Many studies have reported mass mortalities of abalone in several countries, which in turn led to economically substantial losses for abalone farmers (Cai et al., 2007; Iehata et al., 2009; Mulugeta et al., 2009). Among the most common pathogens were member of the bacterial genus *Vibrio*, such as *Vibrio parahaemolyticus* (Shuhong et al., 2004), *Vibrio anguillarum* (Macey and Coyne, 2005), and *Vibrio harveyi* (Jiang et al., 2013a).

1.4. General Approaches

1.4.1. Manufactured pellets and growth hormone

Research to date has investigated several approaches to enhance the growth rate and disease resistance of abalone. Among the available approaches to improve the growth rate are: (1) the development of manufactured pellets with higher protein content (Britz and Hecht, 1997; Bautista-Teruel and Millamena, 1999; Naidoo et al., 2006; Mulvaney et al., 2013), and (2) the use of growth hormones (Taylor et al., 1996; Moriyama and Kawauchi, 2004). However, these approaches have not given significant and consistent results. Mulvaney et al. (2013) compared a high-protein diet (formulated pellet with 32%) to a low-protein diet (macroalgae with ~18%) on the growth of hybrid abalone (*H. rubra* 1814 Leach and *H. laevigata*) and demonstrated that the abalone receiving the formulated pellets grew poorer than abalone fed with the macroalgae. Similarly, Naidoo et al. (2006) showed that *H. midae* fed with a commercial abalone pellet (Abfeed®, 35% CP) had lower growth rate compared to abalone receiving fresh macroalgae (*Gracilaria gracilis* and *Ulva lactuca*). Another study suggested that manufactured pellets which cost 5-7 $AU.kg^{-1}, did not support sustained growth at economically viable rates (Fleming, 2000). In addition, Taylor et al. (1996) injected 5 µg.g^{-1} body weight of four growth hormones (recombinant bovine growth hormone, recombinant porcine growth hormone, somatostatin, and bovine serum albumin)

into *Haliotis kamtschatkana*, and the results indicated that there was no significant difference in weight gain compared to abalone without the hormone injection. These results encourage aquaculture researchers to find alternative approaches to enhance the growth of abalone.

1.4.2. Antibiotics and vaccination

There are two common approaches to control diseases infecting cultivated animals: (1) the use of antibiotic drugs (Friedman et al., 2003; Handlinger et al., 2005) and (2) vaccination (Wu et al., 2011). But these approaches have been criticized due to safety and reliability issues. The massive use of antibiotics, for instance, has been associated with several issues such as inducing antibiotic-resistance pathogens with unpredictable long-term effects on public health (Miranda and Zemelman, 2002); the presence of antibiotic residues in animal flesh for human consumption (Defoirdt et al., 2007; Sahu et al., 2008); and the effect that non-specific targeting may have on beneficial organisms (Sahu et al., 2008). Thus, the World Health Organization (WHO) strictly regulates the use of antibiotics in food industries including.

The development of vaccines has significantly reduced the amount of antibiotic use in aquaculture especially in aquatic vertebrates. However, the reliability of vaccination has been questioned, especially when was applied to those animals with less developed immune systems such as abalone and early stages of aquatic vertebrates (Balcazar et al., 2008; Jiang et al., 2013a). Abalone lack an adaptive immune system, thus obviating the use of vaccination to control disease. As consequence, the search for alternative approaches to improve the growth and survival of abalone is still ongoing research. One approach recommended by The Food and Agriculture Organization (FAO) to solve these problems is the use of probionts (Subasinghe, 1997).

1.4.3. Probionts

WHO defines probionts as *"live microorganisms that when administered in adequate amounts, confer a health benefit on the host"* (FAO/WHO, 2001). The earliest application of probionts was recorded in the 1970s when several probiotic strains were used as feed supplements and demonstrated the improvement of growth or health in terrestrial animals by increasing resistance to diseases (Parker, 1974). Since then, probionts have been applied in many terrestrial organisms including human, pig, cattle, and poultry. The successfulness of probionts in terrestrial organisms leads several researchers to conduct research on the application of probionts in aquatic species.

The first study to report the probiotic application in aquatic species was published in 1980 (Yasuda and Taga, 1980). Thereafter, the interest in the use of probionts for increasing growth and disease resistance of farmed aquatic species has been widely accepted in aquaculture industries due to: (1) probionts are considered environmentally-friendly treatments, (2) the ability to enhance feed digestibility and growth of cultivated species, (3) increased disease resistance of hosts, and (4) suitability for application to animals lacking adaptive immune systems such as invertebrates and early stages of fish (larvae). For these reasons, probionts have been defined as a major area for further research in aquaculture industries by FAO of the United Nations (Subasinghe, 1997).

1.4.3.1. Improving growth

Numerous studies reported that supplementation with probionts separately or in mixtures of several strains increased the growth of teleosts and molluscs including sea cucumber (*Apostichopus japonicus*) (Yang et al., 2014), Indian white shrimp (*Fenneropenaeus indicus*) (Ziaei-Nejad et al., 2006), giant freshwater prawn (*Macrobracium rosenbergii*) (Dash et al., 2016), gilthead sea bream (*Sparus aurata* L.) (Suzer et al., 2008), tilapia (*Oreochromis niloticus*) (Ridha and Azad, 2012), and carp

(*Cyprinus carpio*) (Wang and Xu, 2006). Among the suggested mechanisms for growth enhancement are: (1) the synthesis of enzymes which participates in digestion processes (Ziaei-Nejad et al., 2006; Suzer et al., 2008; Askarian et al., 2011; Ridha and Azad, 2012; Yang et al., 2014); (2) the modification of intestinal environment and gut epithelial cells which allow better solubility and absorption of nutrients (Fjellheim et al., 2010; Hadi et al., 2014), (3) stimulating the expression of two genes involved in growth (Insulin-Like Growth Factor, IGF-I and Growth Hormone, GH) (Shaheen et al., 2014) and (4) improving stress tolerance of cultured animals (Olmos et al., 2011). These possible mechanisms enhance feed assimilation and lead to the increasing of growth performance, Table 1.

The synthesis of digestive enzymes

Various species of bacteria were reported to play important roles in feed digestion due to their ability to excrete digestive enzymes such as amylase, lipase, cellulase, alginate lyase, phytase and protease (Bairagi et al., 2002; Ghosh et al., 2002; Gruchota et al., 2006; Mondal et al., 2008; Ray et al., 2012; Anithajothi et al., 2014; Rajasekaran et al., 2014). For instance, the addition of *Lactobacillus* spp., which could produce protease to larvae of sea bream (*Sparus aurata*, L.) via live feed (artemia and rotifers) showed higher the enzyme activity in the intestinal tract, and gave 2-9 % higher specific growth rate and 13-15% higher survival rate than the control (Suzer et al., 2008). In addition, the supplementation of *Metschnikowia* sp. C14 enhanced the protease and lipase activity in intestinal tract of sea cucumber (*Apostichopus japonicus*) (Yang et al., 2014). Nimrat et al. (2013) reported the enhancement of protease in black tiger shrimp (*Penaeus monodon*) by supplementation of *Bacillus subtilis* and *Enterococcus* sp. These studies suggest that probionts play significant role in helping feed digestion in intestinal tract of cultured animals.

The modification of intestinal environment and gut epithelial cells

Probionts have been documented to modify intestinal environment and gut epithelial cells, which allowed better solubility and absorption of nutrients by cultured animals (Fjellheim et al., 2010). For instance, lactic acid bacteria (LAB) have been reported to produce organic acids which lowered pH of intestinal juice, (Merrifield et al., 2010b). The acidification of the gastric environment has been associated with the enhancement of nutrients' solubility and absorption (Lee et al., 1999). The same idea was proposed by Hadi et al. (2014) in which the supplementation of acid-producing *Enterococcus* sp. through diets decreased intestinal pH and increased nutrient absorption by abalone. Another study by Adeoye et al. (2016) reported that supplementation of probiotics together with exogenous enzymes improved intestinal morphology: higher perimeter ratio, larger diameter and denser microvilli count which lead to the increasing of enterocyte absorptive area resulted in the better growth performance in Tilapia. Similarly, Barroso et al. (2016) presented the supplementation of multi-species probiotics increased the height of intestinal villi in Senegalese sole (*Solea senegalensis*).

Regulating the expression of genes involved in growth and stress

Carnevali et al. (2006) reported that the administration of *Lactobacillus delbrueckii delbrueckii* in juvenile of seabass enhanced the expression of gene involved in muscular growth (IGF-I), while lowering and MSTN mRNA transcription, which was in agreement with the increase of the fish growth. The administration of *Bacillus* mixture upregulated the expression of two genes involved in growth: IGF-I and GH (Shaheen et al., 2014). The administration of *Lactobacillus delbrueckii delbrueckii* in juvenile of seabass lowered cortisol level which is an indicator for stress condition (Carnevali et al., 2006). Similarly, Olmos et al. (2011) also documented the

administration of *Bacillus* sp. lower cortisol level in shrimp. The mechanisms behind this action are still not clear. A study by Neuman et al. (2015) suggests that the bacteria produced hormone, respond to hosts hormones and regulate the expression levels of host hormones.

Table 1. Examples of probiotic supplementation and their mechanisms in enhancing the growth of aquatic animals.

No	Probiotic strains	Suggested mechanism	Reference (s)
1	*Lactobacillus delbrueckii delbrueckii*	Enhanced the expression of gene involved in muscular growth (IGF-I), while lowering and MSTN mRNA transcription in juvenile sea bass	Carnevali et al. (2006)
2	*Bacillus lincheniformis*	Increased amylase activity in the intestinal tract of white shrimp	Ziaei-Nejad et al. (2006); Hu et al. (2008)
3	*Bacillus* spp.	Enhanced protease, amylase, and lipase in the GITs of Indian shrimp	Ziaei-Nejad et al. (2006); ten Doeschate and Coyne (2008)
		Enhancing protease, amylase, and lipase in the GIT of common carp	Wang and Xu (2006); Iehata et al. (2010)
4	*Lactobacillus curvatus* and *Leuconostoc mesenteroides*	Increased amylase, lipase, and protease activity in the intestinal tract of Belunga and Persian sturgeon	Suzer et al. (2008); Askarian et al. (2011)
5	*Bacillus* sp.	Lowered cortisol level in shrimp	Olmos et al. (2011)
6	*Bacillus amyloliquefaciens* *Bacillus subtilis* and *Enterococcus* sp.	Enhanced digestive enzyme activity and improved FCR value in tilapia	Ridha and Azad (2012)
7	*B. amyloliquefaciens* *B. subtilis* and *Enterococcus* sp.	Enhanced trypsin and chymotrypsin in the intestinal tract of Post larva black tiger shrimp.	Nimrat et al. (2013); Hadi et al. (2014)
		Produced trypsin and chymotrypsin in the GIT of black tiger shrimp	Nimrat et al. (2013)
	B. subtilis and *Enterococcus* sp.		
8	*Metschnikowia* sp. C14	Enhanced protease and lipase activity in the intestinal tract of juvenile sea cucumber	Macey and Coyne (2005); Yang et al. (2014)
9	*Lactobacillus* sp.	Enhanced protease, amylase, and lipase activity in the intestinal tract of gilthead sea bream	Suzer et al. (2008); Yang et al. (2014)
10	*Pseudoalteromonas elyakovii* HS1 *Shewanella japonica* HS7	Increase the growth of sea cucumber	Chi et al. (2014)

11	*B. subtilis*, *Bacillus pumilis*, *B. amyloliqueficiens* and *B. licheniformis*	Up-regulated the expression of 2 genes involved in growth: IGF-I and GH	Shaheen et al. (2014)
12	*Vibrio midae* SY9	Produce protease	Huddy and Coyne (2014); (Huddy and Coyne, 2015)
13	*Vibrio* spp.	Increased agarase activity in the GIT of abalone	Faturrahman et al. (2015)
14	*Rhodotorula benthica* D30	Increased amylase, cellulase and alginase activity in the GIT of juvenile sea cucumber	Wang et al. (2015)
15	*B. subtilis*, *B. licheniformis* and *B. pumilus*	Improved intestinal morphology: higher perimeter ratio, larger diameter and denser microvilli count which lead to the increasing of enterocyte absorptive area in Tilapia	Faturrahman et al. (2015); Adeoye et al. (2016);
16	*Lactobacillus plantarum*	Increased protease activity and improved FCR and PER of giant freshwater prawn	Dash et al. (2016).
17	*Virgibacillus proomi* *Bacillus mojavensis*	Enhanced digestive enzyme activity in the intestinal tract of sea bass	Hamza et al. (2016).
18	*Bacillus* sp. *Pediococcus* sp. *Enterococcus* sp. and *Lactobacillus* sp.	Increased the height of intestinal villi in Senegalese sole (*Solea senegalensis*)	Barroso et al. (2016)
19	*B. pumilus* SE5	Produced digestive enzymes	Wang et al. (2015); Ozorio et al. (2016); Yan et al. (2016)
20	*Bacillus coagulans*	Increased the activity of protease, amylase, and lipase in the GIT of freshwater prawn	Gupta et al. (2016); (Ozorio et al., 2016); Yan et al. (2016)
21	*Weissella cibaria*	Produced protease in Siberian sturgeon	Gupta et al. (2016); Hashemimofrad et al. (2016)

GIT: gastrointestinal tract, FCR: feed conversion ratio, PER: protein efficiency ratio

1.4.3.2. Enhancement of disease resistances

Probionts have been also reported to enhance hosts' resistance to disease (Table 2). Among the suggested mechanisms are: (1) producing antimicrobial compounds active against pathogens (Kamei et al., 1987; Westerdahl et al., 1991; Gatesoupe, 1999; Verschuere et al., 2000a; Balcazar et al., 2006; Ringø et al., 2010; Sequeiros et al., 2010; Perez-Sanchez et al., 2011); (2) out competing pathogens for chemical and adhesion sites (Verschuere et al., 2000a; Merrifield et al., 2010a; Geraylou et al., 2014), and (3) stimulating or developing the host's immune systems (Verschuere et al., 2000a; Rawls et al., 2004; Farzanfar, 2006; Chiu et al., 2007; Nayak, 2010a). Due to these capacities, probionts have been considered as a potential way to increase the survival of cultivated animals, especially animals with less-developed immune systems.

A number of probiotic strains were reported to produce various antimicrobial compounds against pathogens such as organic acids, hydrogen peroxide, and bacteriocins (Kamei et al., 1987; Westerdahl et al., 1991; Gatesoupe, 1999; Verschuere et al., 2000a; Balcazar et al., 2006; Ringø et al., 2010; Sequeiros et al., 2010; Perez-Sanchez et al., 2011). Among these antimicrobial compounds, bacteriocins are attracting more interest due to their wide application and being considered as safe substances. Bacteriocins are proteinaceous toxins that can kill pathogens by blocking their metabolic processes or by silencing bacterial virulence by disrupting their quorum sensing (Chu et al., 2011). Common bacteriocins produced by probiotic strains include: (1) nisin which is produced by *Lactobacillus lactis* which effectively suppress the growth of *Aeromonas salmonicida* (Balcazar et al., 2009), (2) carnocin produced by *Carnobacterium* sp. (Ringo and Gatesoupe, 1998; Quadri, 2002), (3) Enterocins, durancin, and mundaticin produced by *Enterococcus faecium* (Lin et al., 2013) and (4) plantaricin, AMP secreted by *Lactobacillus plantarum,* which was reported to inhibits the growth of *A. hydrophila,* more powerful than either chloramphenicol (30 µg) or gentamicin (15

μg) (Quadri, 2002; Cebeci and Gurakan, 2003; Giri et al., 2011). *Lactobacillus paracasei* produces a bacteriocin, a phenolic compound, and *Bacillus* spp. produces lipopeptide antibiotics and surfactants (Giri et al., 2011). Several studies have reported that most of antimicrobial compound-producing bacteria used as probionts belong to members of LAB (Gatesoupe, 1999) due to their GRAS status, generally regarded as safe microorganisms. Therefore, many studies have suggested targeting members of LAB for screening of antimicrobial compound production, as probiotic candidates.

Table 2. Examples of probiotic application and their mechanisms in enhancing aquatic animals' resistance to pathogens.

No	Probiotics strains	Suggested mechanism	Reference (s)
1	*Carnobacterium divergens*	Producing antimicrobial compounds which protected cod fry from *V. anguillarum*	Gildberg and Mikkelsen (1998)
2	*Aeromonas caviae*	Protecting *Artemia* from *Vibrio alginolyticus* due to competitive exclusions	Verschuere et al. (2000b)
3	*Lactobacillus rhamnosus*	Protecting rainbow trout against *A. hydrophila* through antimicrobial compound production, competitive exclusion, and immune modulations.	Nikoskelainen et al. (2001a); Panigrahi et al. (2005)
4	*Lb. plantarum*	Increasing resistance of white shrimp against *V. alginolyticus* by enhancing haemocyte counts, phenol oxidase and phagocytic activities.	Chiu et al. (2007)
5	*Leuconostoc mesenteroides CLFP 196* and *Lactobacillus plantarum CLFP 238*	Protecting rainbow trout against lactoccosis	Vendrell et al. (2008)
6	*Pediococcus acidilactici*	Protecting shrimps from *V. nigripulchritudo* by immune stimulation	Castex et al. (2008)
7	*Enterococcus faecium* and *Streptococcus phocae*	Protecting shrimp from vibriosis by competitive exclusion and antimicrobial compound production	Swain et al. (2009)
8	*Pediococcus acidilactici*	Enhanced total antioxidant status in shrimp	(Castex et al., 2009)
9	*Enterococcus faecium* MC13	Protecting carp from *Aeromonas hydrophila*	Gopalakannan and Arul (2011)
10	*Lactobacillus. acidophilus*	Protecting African catfish, *Clarias gariepinus*, from *A. hydrophila* and *Streptococcus agalactiae* by stimulating the production of haematocrits and immunoglobulin G (Ig).	Al-Dohail et al. (2011)

11	*Lactobacillus paracasei* subsp. tolerans F2	Protecting rainbow trout against *Yersnia ruckeri* and *A. hydrophila* by antimicrobial compound production	Sica et al. (2012)
12	*Lb. plantarum* and *B. subtilis*	Protecting European sea bass larvae against *V. anguillarum* by producing antimicrobial compounds productions	Touraki et al. (2012)
13	*Shewanella olleyana* WA65 *Shewanella colwelliana* WA64	Enhanced cellular and humoral immune response; hemocytes, respiratory burst activity, serum lysozyme activity, resulted in higher survival rate of abalone against *V. harveyi*	Jiang et al. (2013a)
14	*Lactococcus lactis* D1813	Immunomodulatory role in kuruma shrimp by enhancing the production of interferon (IFN) and antimicrobial compounds.	Maeda et al. (2014)

In addition, protection capacity of probionts to cultivated animals have been reported due to outcompeting bacterial pathogens for ecological niches by preventing bacterial pathogens from adhering to infection sites such as intestinal tracts (Verschuere et al., 2000a). A study by Verschuere et al. (2000b) confirmed that *Aeromonas caviae* protected *Artemia* sp. against *Vibrio proteolyticus* due to blocking the adherence of the pathogen, rather than secretion of extracellular antimicrobial compounds. Other studies explained that adhesion capacity is considered to be an initial stage for pathogens to infect animal hosts (Horne and Baxendale, 1983; Coquet et al., 2002), therefore probionts with good adhesion activity could prevent the adherence of, and infection by bacterial pathogens in cultured animals (Nikoskelainen et al., 2001b; Ouwehand et al., 2001; Servin and Coconnier, 2003; Vendrell et al., 2009).Theoretically, the adhesion can be based on host-microbe specific interaction or host-microbe non-specific interactions (Balcazar et al., 2009; Sica et al., 2012). In specific mechanisms, the adhesion is based on physicochemical factors such as carbohydrate-specific molecules on the bacterial cell surface (Balcazar et al., 2009). Nonspecific adhesion is based on the involvement of adhesion molecules on the bacteria surface and receptor molecules on the fish mucosal surfaces such as non-covalent bonding and hydrophobic interactions (Verschuere et al., 2000a; Balcazar et al., 2009; Sica et al., 2012). Due to the critical roles of adhesion activity, many studies screen probiotic candidates based on their adhesion capacity to common infection sites such as intestinal mucus (Ouwehand et al., 2001; Servin and Coconnier, 2003; Geraylou et al., 2014) or fish tissues (Vendrell et al., 2009).

1.5. Probiotic application in abalone

Studies which investigate probiotic application in abalone species are still very limited, compared to other farmed aquatic species. Up to now, there are only few studies which have investigated the effect of probiotic supplementation on the growth or disease resistance of

abalone species, Table 3. In attempt to improve growth rate, most studies tested the supplementation of alginate lyase-producing bacteria (ten Doeschate and Coyne, 2008; Hadi et al., 2014) to enhance feed digestibility of abalone. This may be based on previous study by Sawabe et al. (1995) who reported that one of building structures of seaweed (the most preferred natural diet) with high energy content, polyguluronate G (polyG), could not be digested by abalone. A few years later, a study conducted by Erasmus et al. (1997) reported that several bacterial strains could synthesize an enzyme which was able to digest the polysacharide blocks, and this enzyme is known as alginate lyase. Since then, number of studies have used alginate-lyase producing bacteria (separately or in mixtures with other enzyme-producing bacteria) as feed supplements for abalone. The results were quite promising wherein growth enhancment was caused not only by increasing activities of the digestive enzymes, but also by increasing solubility and absorption of nutrients. A study by Hadi et al. (2014) reported that supplementation of acid-producing *Enterococcus* sp. improved nutrient solubility by lowering pH of intestinal tract of abalone and resulted in growth improvement.

In addition, to the author's knowledge, only one study has specifically investigated the use of probiotic supplementation on the diseases resistance of abalone (Table 3). The study showed that supplementation of two probionts (*Shewanella colwelliana* WA64 and *Shewanella olleyana* WA65) enhanced hemocytes, respiratory burst activity, and serum lysozyme activity of abalone (Jiang et al., 2013a). Furthermore, the probiotic administration, improved the survival rate of abalone by 30 % after being challenged with *V. harveyi*. This study indicates that probionts may be a useful method to improve disease resistance of abalone, although more studies still need to give higher survival rate.

Table 3. The supplementation of probiotic strains and their effects on the growth performance of abalone

No	Probiotic strains	Effect	Source of strain	Application method	Abalone life stage	Reference
1	*Vibrio midae*	Increased the growth of abalone by enhancing protease and amylase activity in the intestinal tract of *H. midae*	GITs of *H. midae*	Mixture with commercial feed	Juvenile (20mm) and adult (67mm)	Macey and Coyne (2005)
2	*Pseudoalteromonas* sp. strain C4	Increased alginate lyase, resulted in better digestion of kelp in *H. midae*	GIT of *H. midae*	Mixture with feed (kelp cake)	Juvenile (25mm)	ten Doeschate and Coyne (2008)
3	*Pediococcus* sp. Ab1	Increase alginate lyase activity and volatile short-chain fatty acids (SCFAs) and improve the growth of *H. gigantea*	GIT of *H. gigantea*	Mixture with commercial feed.	Adult (9 months)	Iehata et al. (2010)
4	A mixture of *Vibrio* sp. C21-UMA, *Agarivorans albus* F1-UMA and *Vibrio* sp. F15-UMA	Increased digestive enzyme activity (e.g., alginate lyase and protease) which resulted in 8% higher in shell length and 16% in wet weight of *H. rufescens* compared to the growth rate in the control. Improved 3% in survival rate compared to the survival in the control.	GIT of *H. rufescens*	Mixture with macroalgae	Juvenile (19mm), and adult (36mm)	Silva-Aciares et al. (2011)
5	*Shewanella colwelliana* WA64 *S. olleyana* WA65	Improved disease resistance of *H. discuss hannai Ino* against *V. harveyi* by enhancing cellular and humoral immune response	GITs of *H. discuss hannai Ino*	Mixture with commercial pellet	Adult (8 g)	Jiang et al. (2013a)

6	*Exiguobacterium* JHEb1, *Vibrio* JH1, and *Enterococcus* JHLDc	Increased alginate lyase and protease activity, lead to better digestion and absorption of feed as well as the growth of *H. iris*	GITs of *H. iris*	Mixture with commercial pellets	Juvenile (20-30mm)	Hadi et al. (2014)

Although, the studies demonstrated that probiotic inclusions could significantly increase the growth or enhance disease resistance of abalone, there are many aspects which still need to be studied to optimize the effect of probiont applications in abalone industries.

1.6. Research gaps

There are several issues which have been faced in applying probiont to farmed abalone, including viability and stability of probionts in the GIT of abalone. Previous studies indicated that the administered probionts persisted for a very short time and disappeared completely from the GITs only few days after the administering process were terminated (Macey and Coyne, 2006; Iehata et al., 2009; Silva-Aciares et al., 2011). These may be due to several possibilities:

1. Probionts were isolated from terrestrial organisms. For example, *Lactobacillus* sp. and *Enterococcus* sp. originated from horse manure, and rice bran which were administered into *H. gigantea* disappeared from the abalone intestine within four days after supplementation was terminated (Iehata et al., 2009). Similar phenomena were observed from other aquatic species where terrestrially-isolated probionts showed low viability in the GITs of aquatic species (Nikoskelainen et al., 2001b; Carnevali et al., 2004; Balcázar et al., 2007; panigPicchietti et al., 2007; Iehata et al., 2009; Reda and Selim, 2015). The reason may have been because these probionts have difficulty in adapting to the intestinal environmental conditions of the aquatic animals. Several studies recommended to isolate probionts for aquaculture purposes from indigenous bacterial communities of aquatic species (e.g., Fjellheim et al., 2010; Spanggaard et al., 2001). Thus, screening of probiotic candidates for their potential capacity to tolerate the low pH of stomach, to survive digestion by

surfactants such as bile salts and to tolerate gastric enzymes such as trypsin and pepsin should be investigated.

2. Screening methods in probiotic selection was only based on their ability to synthesize desired beneficial compounds such as digestive enzyme or antimicrobial compounds. Meanwhile, other important parameters such as viability in GITs of cultivated animals were not studied. Hadi et al. (2014) for instance, screened and selected probiotic candidates only based on enzyme production and tolerance to low pH. While, susceptibility of probiotic candidates to other surfactants in the GITs such as bile salts and to gastric enzymes (e.g., pepsin and trypsin) or to the different environment such as the salinity of the rearing water were not investigated. Several studies concluded that bile salt and gastric enzymes can affect the viability of bacteria in the GIT of cultivated animals (Panigrahi et al., 2005; Gebara et al., 2013; Geraylou et al., 2014; Saito et al., 2014). Thus, screening of probiotic candidates for their potential capacity tolerate low pH of stomach and surfactants such as bile salts as well as gastric enzymes such as trypsin and pepsin should be investigated.
3. In addition, several studies only rely on *in vitro* screening processes to select probiotic candidates (Vine et al., 2004; Hagi and Hoshino, 2009; Lin et al., 2013; Geraylou et al., 2014). Meanwhile, several studies suggested that neither positive nor negative *in vitro* results may predict the actual effect of *in vivo* studies (Verschuere et al., 2000a; Balcazar et al., 2006). Thus, more comprehensive studies from isolation sources, *in vitro* screening and characterization, followed by *in vivo* study are required to select probiont candidates.

Acknowledging these issues, the present study was designed to find probiotic candidates which are not only producing digestive enzyme or antimicrobial compounds but

also have better viability and stability in the GIT of abalone, as well as having significant contribution in growth or survival rate of live abalone.

1.7. Objectives

Overall, the present study aimed to isolate and characterize probiotic candidates from the GITs of teleosts and molluscs. Specificobjectives of this present study are:

(1) Isolation of endogenous bacteria from the GIT of teleosts and molluscs which have capacity to produce important digestive enzymes (protease, amylase, lipase, cellulase, and alginate lyase), and/or bacteria with capacity to produce antimicrobial compounds against several aquatic pathogens including *Vibrio*, a genus commonly associated with abalone mortalities.

(2) Investigation of the viability and stability of the candidate probionts within the GIT of abalone by *in vitro* exposure to simulated GIT conditions such as low pH in stomach, gastric enzymes, and bile salts.

(3) Investigation of probiotic effects on the growth and survival of juvenile abalone by *in vivo*.

An overview of the whole study is presented in Figure 1.

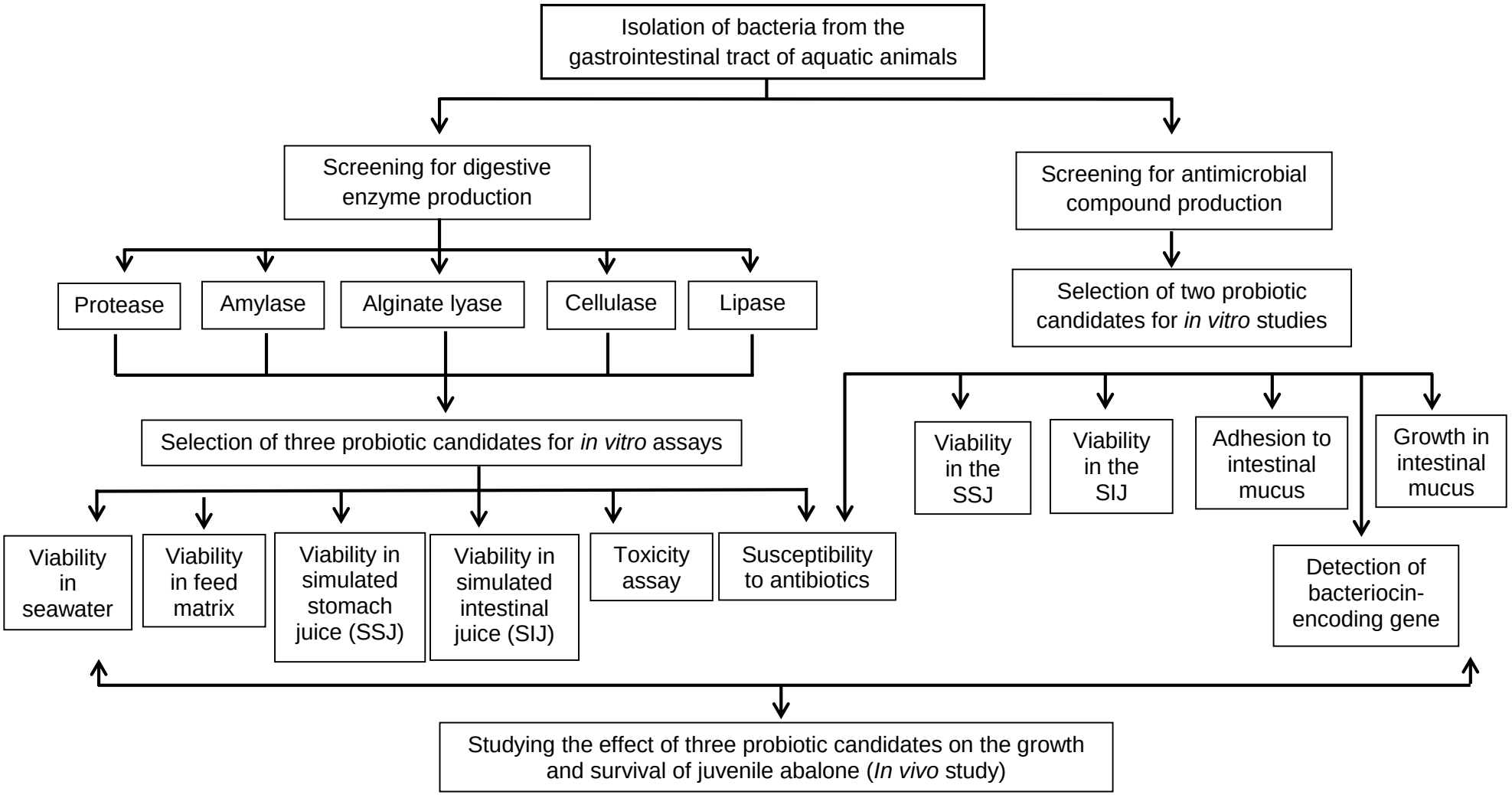


Figure 1. An overview of the whole study

1.8. Thesis outline

This thesis consists of six chapters. Chapter 1 begins with a general introduction. It gives an overview of, and background to, abalone aquaculture, the major constraints which are still faced and the alternative solutions to the constraints of abalone aquaculture. In addition, this chapter discusses more about probionts as a potential approach to increase the growth and disease resistance of cultivated animals. Current applications of probionts in abalone aquaculture are also described, followed by the gaps in previous studies, concluding with the approaches used in this present study to improve the growth and survival rate of abalone aquaculture.

Chapter 2 details the isolation and identification of intestinal bacteria from the GIT of aquatic species including abalone, seabream, Atlantic salmon, mussel, and other fish collected from wild environments. This present study targeted those bacteria which have potential capacity to synthesize digestive enzymes (protease, cellulase, amylase, lipase, amylase, and alginate lyase) based on a capacity to utilize several substrates such as casein, gelatin, sodium alginate, starch, tributyrin and carboxymethyl cellulose (CMC) for preliminary screening. Meanwhile, members of LAB were targeted for antimicrobial compound-producing bacteria. LAB were selected because LAB have GRAS status, and well known for their capacity to produce various antimicrobial compounds against various bacterial pathogens. Representative bacterial isolates from both bacterial groups were identified phenotypically and validated molecularly based on their 16S rRNA gene sequence.

Chapter 3 describes the screening of the acquired intestinal bacteria and quantified their digestive enzyme activity based on their capacity to degrade indicator substrates: casein and gelatin for protease activity, sodium alginate for alginate lyase activity and

carboxymethyl cellulose for cellulase activity. Bacterial isolates exhibiting the strongest enzyme activity from each enzyme group were selected for further studies, including viability and stability in feed matrix stored at low temperatures, survival rate in rearing water, viability to the simulated intestinal juice especially low pH in stomach, surfactants such as bile salts and gastric enzymes (e.g., pepsin and trypsin), and followed by an *in vivo* toxicity assay to juvenile abalone.

Chapter 4 describes the screening processes of the intestinal bacteria for antimicrobial-compound productions against eight bacterial pathogens. The screening was only performed to 206 LAB. Two LAB which displayed the broadest spectrum against vibrios (as the most common pathogens in marine aquatic species including abalone) were chosen for further characterizations, including their viability in simulated stomach and intestinal conditions, capacity to adhere to and grow in intestinal mucus. In addition, capacity of the 2 LAB strains to produce bacteriocin like inhibitory substance (BLIS) was investigated by detecting the presence of pediocin PA1-encoding gene, as one of the most common bacteriocin produced in genus Pediococcus.

Chapter 5 presents an *in vivo* study testing if probiotic candidate strains selected based on their performance in *in vitro* studies (Chapters 3 and 4) had beneficial effects on the growth and survival rates of abalone. Overall, three probiotic candidates were selected and fed to juvenile abalone, *H. asinina*, for 62 days, via natural diet of abalone, *Gracillaria* sp. Subsequently, the effect of probiotic supplementation on the growth and survival rates of the juvenile abalone was compared.

Chapter 6 summarises the results and findings from the present study, followed by a general discussion that includes the limitations of this study, and recommended future work.

Chapter 2 : Isolation and Identification of Bacteria from The Gastrointestinal Tracts of Teleosts and Molluscs

2.1. Introduction

Aquatic animals harbor diverse microbial populations including bacteria in their GITs. These bacteria come from rearing water through drinking or diets during feeding (Trust et al., 1979; Cahill, 1990; Amin, 2010). Some of the ingested bacteria are only transient in the GITs due to being unable to tolerate harsh environmental conditions in the GITs such as low pH, bile-salt content, and gastric enzymes. Other bacteria may be able to colonize the GITs, because of their ability to tolerate the harsh environmental conditions (Onarheim and Raa, 1990; Ringø et al., 1995). Previous studies counted a total number of bacteria living in the GITs of aquatic animals was much higher than in their surrounding environments (e.g., rearing water, animal skin or gills), due to the rich nutrient content in the GITs (Cahill, 1990; Ringø et al., 1995). The high nutrients in the GITs are coming from ingested diets, digestive secretions and fragments sloughed off from the mucosal epithelium (Bairagi et al., 2002). In return, numerous microorganisms contribute to their hosts by producing various beneficial substances including digestive enzymes, vitamins, growth hormone-stimulating substances (Prieur et al., 1990; Askarian et al., 2011; Ray et al., 2012) and antimicrobial compounds active against pathogens (Balcazar et al., 2008; Askarian et al., 2009; Sequeiros et al., 2010). Due to these capacities, the isolation of intestinal bacteria that can benefit their hosts, by improving growth rate or disease resistance against pathogens has increased considerably in the last few decades.

Digestive enzyme-producing bacteria can be used as feed supplements to enhance feed digestibility and growth of cultured animals. Among the digestive enzymes are: protease, amylase, cellulase, and lipase (Bairagi et al., 2002; Ray et al., 2010; Rajasekaran et al., 2014). Besides those enzymes, abalone require another digestive enzyme called alginate lyase (Sawabe et al., 1998; Tanaka et al., 2003); (Miyake et al., 2003). The enzyme is vital to break down alginate content found in macroalgae, which are common abalone

feeds. Meanwhile in terms of diseases-controlling agents, studies mostly focus on Gram-positive bacteria, such LAB. LAB are well known for producing various antimicrobial compounds such as bacteriocins (Verschuere et al., 2000a; Lin et al., 2013), bacteriolytic enzymes, hydrogen peroxide (Verschuere et al., 2000a) and organic acids (Goncalves et al., 1997; Vazquez et al., 2005). In addition, many members of LAB are well known for their ability to outcompete pathogens in nutrient uptake or for adhesion sites in gut mucosa (Nikoskelainen et al., 2001b), having high resistant to the harsh environmental conditions, and are able to stimulate the hosts' immune systems (Panigrahi et al., 2005; Chiu et al., 2007). Furthermore, LAB are generally recognized as safe (GRAS) microorganisms (Hwanhlem et al., 2014).

To date, the application of the beneficial bacteria generally called probionts has been increasingly popular in cultured teleosts and molluscs. However, some studies indicated that viability of probiotic strains in the GITs of aquatic animals is very low, resulted in a decrease of probiotic contribution to the hosts. Other authors suggested that the low viability was due to difficulties in adapting to the harsh conditions of GITs, as these probiotic strains are isolated from terrestrial organisms (Nikoskelainen et al., 2001b; Iehata et al., 2009; Reda and Selim, 2015). Therefore, it has been suggested that the best place to search for probiotic candidates for aquaculture animals is from those bacteria which associated with the GITs of aquatic species (Fjellheim et al., 2010), acknowledged as indigenous bacteria. These indigenous bacteria are presumed to be more adaptable to the ecological niche of GITs, and consequently may have a greater chance of colonizing guts and conferring health benefits to their hosts.

This research aimed at the isolation and identification of endogenous enzyme-producing bacteria and LAB and from the GITs of teleosts and molluscs. Two separate

isolation strategies were used to target 1) bacteria which produce protease, cellulase, amylase, lipase, and alginate lyase, and 2) members of the LAB as bacterial candidates for controlling bacterial pathogens. Acknowledging the high number of intestinal bacteria, this study used an enrichment culture method to isolate enzyme-producing bacteria. Meanwhile, the isolation of LAB was performed by using direct spreading methods and also enrichment culture method.

2.2. Materials and Methods

2.2.1 Samples of teleosts and molluscs

This study used a total of 155 teleosts and molluscs collected from different rearing environments, including a recirculating aquaculture system (RAS), a flow-through aquaculture system (FTS) and estuarine and marine waters around Tasmania, Table 4. The animal species included Atlantic salmon (*Salmo salar*), mullet (*Trachystonia petardi*) weighing 200.4±12.2 g and brown trout (*Salmo trutta*) weighing 75.0±7.0g (140.0±44.2 cm), which were collected from a RAS at the Aquaculture Centre, Institute for Marine and Antarctic Studies (IMAS) Launceston, University of Tasmania. In addition, three life stages of hybrid abalone (*Haliotis rubra* x *H. laevigata*): early juvenile (3.9±0.4 mm), late juvenile (17.5±1.2 mm) and adult stage (82.8±3.6 mm) reared in a flow-through aquaculture system, were obtained from Abalone Tasmania (Abtas), Clarence Point, Tasmania. Furthermore, wild species such as seabream (*Sparus sarba*), trevally (*Pseudocaranx georgianus*) and Australian salmon (*Arripis trutta*) were caught from Scamander Estuary. Additionally, several marine species including jack mackerel (*Trachurus declivis*), gurnard perch (*Neosebastes pandus*), longfin pike (*Dinolestes lewini*), tiger flathead (*Neoplatycephalus richardsoni*) and blue mussels (*Mytilus edulis*) weighing 14.03±1.15 g were obtained from a recreational fishing around Tasmania.

Table 4. Samples of teleosts and molluscs

Closed Environment	Semi-closed environment	Open/wild environment	
RAS	**FTS**	**Estuarine**	**Marine**
- 15 Atlantic salmon (*Salmo salar*) - 4 mullet - 4 brown trout	Abalone: - 30 early juveniles - 30 late juveniles - 20 adults	- 15 seabream - 3 trevally - 4 Australian salmon	- 3 jack mackerel - 4 gurnard perch, - 5 longfin pike - 2 tiger flathead - 20 blue mussels

RAS=recirculating aquaculture system, FTS: flow-through aquaculture system.

The protocol for killing live fish sourced from the RAS system was approved by the Ethics Committee for the Use of Animals for Scientific Purposes, the University of Tasmania (Code: A00012135). In brief, fish samples were fasted for 24 h to empty their gastrointestinal contents. Afterwards, the fish were taken out by a scope net and immersed in anaesthetic solution (AQUI-S®, New-Zealand Ltd) at a concentration of 30 mg L^{-1} for 15 min for anesthetization. After the opercula movement had ceased, the fish were killed by spiking the hindbrain. The GIT was then removed aseptically and placed individually in sterile tubes. The tubes were stored in an ice box and processed for bacterial isolation within 1-2 h.

Animal ethics approval for killing invertebrates, including abalone and blue mussels, was not required. Briefly, these invertebrate animals (abalone and mussels) were transported live in an ice box within approximately 2 h to the Aquatic microbiology laboratory. The animals were then anesthetized at 30 mg L^{-1} AQUI-S of seawater. Afterwards, the GITs of the animals were aseptically removed and placed in the ice box until further use. In addition, several wild-caught fish such as seabream, trevally, Australian salmon, and other marine species were obtained from recreational fishing. Freshly-caught fish were killed by spiking quickly into the hindbrain. Afterwards, the GIT was aseptically excised, rinsed three times

with sterile 0.85% normal saline solution (NSS) (BR00536, Oxoid, UK) and placed in a tube and stored on ice. The GITs were then brought to the Aquatic microbiology laboratory, Human Life Science, for further studies within 2 h.

2.2.2. Isolation of intestinal bacteria

Isolation of endogenous bacteria from the GITs of animal samples were performed using an enrichment culture and a direct spreading culture method.

2.2.2.1. Direct spreading culture technique

One-two g of GIT was homogenized in two-four mL 10 mM phosphate buffer saline (PBS), pH 7.2, with a stomacher (Lab Blender400). Subsequently, serial tenfold dilutions (10^{-1} – 10^{-4}) of gut homogenate were made, and 100 µL from each dilution was spread onto the surface of enzyme-selective agar (Table 5) and de Man Rogosa Sharpe (MRS) agar (CM0361, Oxoid, UK) plates containing 10 g L^{-1} calcium carbonate ($CaCO_3$) (A827266, Merck Germany) as an indicator (Lin et al., 2013) and M17 agar (CMO785, Oxoid, UK). The enzyme-selective plates were incubated at room temperature aerobically for 4 days. It was generally assumed that bacteria which could grow on the agar plates with the specific medium would have the capacity to produce enzymes to degrade the substrate in the medium. Meanwhile, MRS-agar plates were incubated anaerobically in an anaerobic jar at room temperature (22 ± 2.0 ^{o}C) for seven days.

The distinct colony phenotypes (size, pigmentation, surface, margin, and opacity) were picked off individually and streaked repeatedly onto the tryptone soya agar (TSA) (CM0131, Oxoid, UK) plates or MRS agar plates until pure isolates were obtained. Stock of purified isolates were further stored in MRS broth (CM0359, Oxoid, UK) medium for LAB or TSB for enzyme-producing bacteria supplemented with 15 % glycerol stock at -20 ^{o}C.

2.2.2.2. Enrichment culture technique

The enrichment culture method was performed according to a protocol developed by Kim et al. (2010) with slight modification. Briefly, five g of pooled GITs based on animal species were mixed with 45 mL sterile seawater (32 ppt), and homogenized in a stomacher (Lab-Blender 400, Townson & Mercer Pty. Ltd, N.S.W). 10-20 mL of the homogenate was then inoculated into 500 mL sterile seawater containing either 2.5 % casein (2330, Ajax Chemicals, Australia) and 2.5 % gelatin (1080, Ajax Chemicals, USA) for protease detection; 5 % sodium alginate (SL117, Chem-Supply) for alginate-lyase activity; 5 % carboxymethyl cellulose (C-4888, Sigma Chemicals, USA) for cellulase activity; or 5 % starch (S-7260, Sigma Chemicals, USA) for amylase and 5 % tributyrin (103111, MP Biochemicals) for lipase. Meanwhile, the homogenized gut was inoculated in MRS broth for isolating LAB.

After 14-day incubation, 1 mL aliquots were serially diluted and 100 µL of each dilution was spread on agar plates with composition as presented in Table 5. The plates were afterward incubated at room temperature aerobically for 2 days. It was generally assumed that bacteria which could grow on the agar plates with the specific medium would have the capacity to produce enzymes to degrade the substrate in the medium. Well-separated colonies with different morphological appearance (colour, surface, margin, and opacity) (from each selective media) were transferred by streaking repeatedly on agar plates until pure. The pure colonies were preserved in 15 % glycerol stock (24388.295, VWR Belgium) and stored at -20 ^{o}C.

Table 5. Selective media for isolation of enzyme-producing bacteria using an enrichment culture method.

Targeted enzyme	Medium Composition	References
Protease	0.2 % (w/v) yeast extract (LP0021, Oxoid UK), 0.3 % (w/v) casein, 0.2 % (w/v) $MgSO_4$ (A648487, BDH England), 0.1 % (w/v) glucose (10117, Merck Australia), 0.5 %(w/v), 0.3 % (w/v) gelatin, 0.001 % (w/v) $FeSO_4$ (28400, BDH Australia), 0.02 % (w/v) K_2HPO_4, and 1.5 % (w/v) bacteriological agar (LP0011, Oxoid UK).	Modified protocol of Li et al. (2008).
Amylase	Starch (0.1 %), peptone (LP0037, Oxoid) (0.05 %), $K_2HPO_4.2H_2O$ (0.02 %), $MgSO_4.7H_2O$ (0.005 %), $FeCl_3$ traces, glycerol (0.1 %) adjusted pH at 7.0. and bacteriological agar (1.5 %).	Modified protocol of Hadi et al. (2014); Rajasekaran et al. (2014)
Cellulase	KH_2PO_4 (0.05 %), $MgSO_4$ (0.025 %), carboxymethyl cellulase (CMC) (0.2 %), agar powder (1.5 %), Congo-Red (0.02 %), and gelatin (0.2 %); adjusted pH to 6.8–7.2, and bacteriological agar (1.5 %).	Modified protocol of Gupta et al. (2012)
Lipase	Peptone (0.5 %), yeast extract (0.3%), tributyrin (1 %) and agar (2 %), adjusted pH to 7.0, and bacteriological agar (1.5 %).	Modified protocol of Sirisha et al. (2010)
Alginate lyase	Polypeptone (0.05 %), yeast extract (0.03 %), sodium alginate (0.2 %), $(NH_4)_2SO_4$ (0.2 %), KH_2PO_4 (0.1 %), and $MgSO_4$ $7H_2O$ (0.05 %). bacteriological agar (1.5 %), and bromothymol blue (20020, BDH Australia) (0.003 %).	Modified protocol of Kim et al. (2009); Nakamura (1987).

2.2.3. Preparation of glycerol stock

Glycerol stock (50 %) was prepared by mixing 50 mL glycerol (24388.295, VWR Belgium) in 100 mL broth media either MRS or TSB. The mixed media were then vortexed and sterilized by autoclaving. Then, 400 µL of the sterilized media was pipetted into 1.8 mL cryovial tube. 800 µL of bacterial isolates (result of 2.2.1-2.2.2) grown overnight was added to the cryovial tube in order to get 20 % glycerol stock. Thereafter, the glycerol stock was stored at -20^{o}C, until further used.

2.2.4. Identification of bacterial isolates

2.2.4.1. Phenotypic assays

Phenotypic assays were performed according a protocol developed by Burke (2011). These assays were colony appearance, Gram-staining, cell shape and arrangement, oxidase, catalase, and glucose fermentation. In brief, each bacterial isolate was cultured on either MRS agar for LAB or TSA for other bacterial isolates. After 24 h incubation, bacterial colony which grew was observed to determine the colony appearance.

Gram staining: a small drop of distilled water was placed on the centre of a sterile microscope slide. Then, a sterilized loop was touched on a fresh and young colony and emulsified on the slide. After the smear was dry, bacteria on the slide was fixed by passing it three times through the flame (the total period of heating was not more than two sec). Thereafter, the slide was flooded with crystal violet for 30 sec, with iodine for 60 secs, alcohol-acetone for 5 sec, and by safranin flooding for 15 sec. The slide was washed with tap water and carefully dried with tissue and viewed under microscope. Gram (+) appeared purple while Gram (-) appeared pink.

Catalase test: an end of a sterile capillary tube was used to touch to the colony of the organism. Immediately, the capillary tube was placed into a tube containing 3

% hydrogen peroxide (H_2O_2) solution. Catalase positive was determined by immediate production of gas bubbles (oxygen). *Staphylococcus aureus* was used as control positive and *Streptococcus* sp. as control negative.

Oxidase test: A small place of filter paper was placed in a petri dish and soaked with 1 % tetra methyl-p-phenylenediamine (T3134-5G, Sigma Chemicals, USA). Then, a fresh young colony was picked with a clean and sterilized tooth pick and placed on the filter paper. A positive oxidase was indicated by the immediate appearance of a dark blue or black colour (within 10 sec).

Glucose fermentation: A medium was prepared by dissolving several chemical compounds (NaCl 5 g, peptone 10 g, 10 mL of 0.2 % aq. Bromo cresol purple, and 900 mL distilled water), with pH of 7.2-7.4. The medium was then sterilized by autoclaving at 115 ^{o}C for 20 min. Afterwards, 90 mL of 10 % sugar solution was added to the sterilized medium. Afterwards, an inverted Durham tube was placed into the bottle (no bubble should be allowed in the Durham tube). A fresh young colony was picked with sterile loop and washed in the medium. The formation of yellow solution after 24 h incubation, indicated that the bacterial isolate was positive glucose fermentation.

2.2.4.2. Genotypic identification

The genotypic identification was performed using a colony polymerase chain reaction (PCR) with a thermal cycler (GeneAmp PCR system 9700, Applied Biosystem, Forster City, CA, USA) to 76 isolates (14 enzyme-producing bacteria and 62 LAB). Briefly, tested bacteria were subcultured individually in MRS broth or Muller Hinton broth for 24 h. A single pure colony was plated on either MRS or MH agar (CM0337, Oxoid, UK). After 24 h incubation, a single pure colony was picked with a micropipette tip and added into 30

µL of master mix as DNA template. The master mix was prepared with composition as below: 4.0 µL of 5x buffer HF buffer, 2 µL $MgCl_2$, 2.0 µL of 10 µM deoxynucleotide triphosphate (dNTP), 2.0 µL of 10 µM forward primer, 2.0 µL of 10 µM reverse primer, 0.3 µL of 4 U/µL *Taq* polymerase (201203, QIAGEN), and 17 µL of H_2O. The oligonucleotide primers used for the bacterial 16S rRNA gene were: 27F (5'-AGA GTT TGA TCC TGC CTC AG-3') and 907R (5'-CCG TCA ATT CCT TTG AGT TT-3') (Lane, 1991) based on the results of Perez et al. (2011). The thermocycle program was as follows: initial denaturation for 10 min at 95 ^oC, 35 cycles of denaturation at 94 ^oC for 15 sec, hybridization at 50 ^oC for 1 min, elongation at 72 ^oC for 1 min, post-elongation at 72 ^oC for 10 min, and cooling down at 10 ^oC for 3 min. After cycling, the PCR products were examined by electrophoresis on a 1% agarose gel, stained with GelRED ™ (Biotium) and visualized under UV light. The 16S rRNA gene amplicons were then purified with UltraClean PCR Clean-up kit (MO BIO) according to manufacturer's instructions and the DNA yield and purity was measured by Qubit ^{TM} Fluorometric Quantification. Then, sufficient purified DNA fragments were sent to an automated DNA sequence provider. The identity of sequenced isolates was compared to published sequences using the BLAST search algorithm (GenBank National Centre for Biotechnology Information, NCBI) with data base 16S ribosomal RNA sequence (Bacteria and Archea) with NRxx as reference number.

2.2.4.3. Phylogenetic diversity

Firsty, an alignment was build from the 14 sequences of enzyme-producing bacteria using *Geneious Alignment*. Then, the sequences were trimmed at the end to have the same length, and used as an input of phylogenetic tree using the Neighbor-Joining method. The constructed tree was built with 100 bootstrap replicates, and this was done only for the phylogenetic tree of digestive enzyme-producing bacteria. In addition, another phylogenetic

tree was constructed using 62 LAB sequences (without bootstrap), and the closest known sequences of bacteria published in GenBank using Geneious software version 5.3.6.

2.3. Results

2.3.1. Enzyme-producing bacteria

A total of 24 bacterial isolates indicating extracellular enzyme productions were isolated from the GITs of Australian salmon (n=1), sea bream (n=1) and hybrid abalone (n=21), Table 6. All bacterial strains were isolated using the enrichment culture method. While no enzyme-producing bacteria could be isolated using the direct spreading culture method. The enzyme production was detected from the presence of clearance zones around bacterial colonies in either CMC-Congo Red agar for cellulase activity respectively (Figure 2a**)** or casein-agar for protease activity and (Figure 2b). In addition, the alginate lyase activity was observed from the colour changing, from yellow to blue on the sodium alginate agar (Figure 2c & 2d**)**. However, no bacterial isolates which produce either lipase or amylase could be isolated from this present study.

Table 6. The number of enzyme-producing bacteria isolated from the GITs of abalone, sea bream and Australian salmon.

No	Enzyme	Abalone	Sea bream	Australian salmon
1	Protease	10	1	-
2	Cellulase	6	0	1
3	Alginate lyase	6	-	-
4	Amylase	-	-	-
5	Lipase	-	-	-
	Total	21	1	1

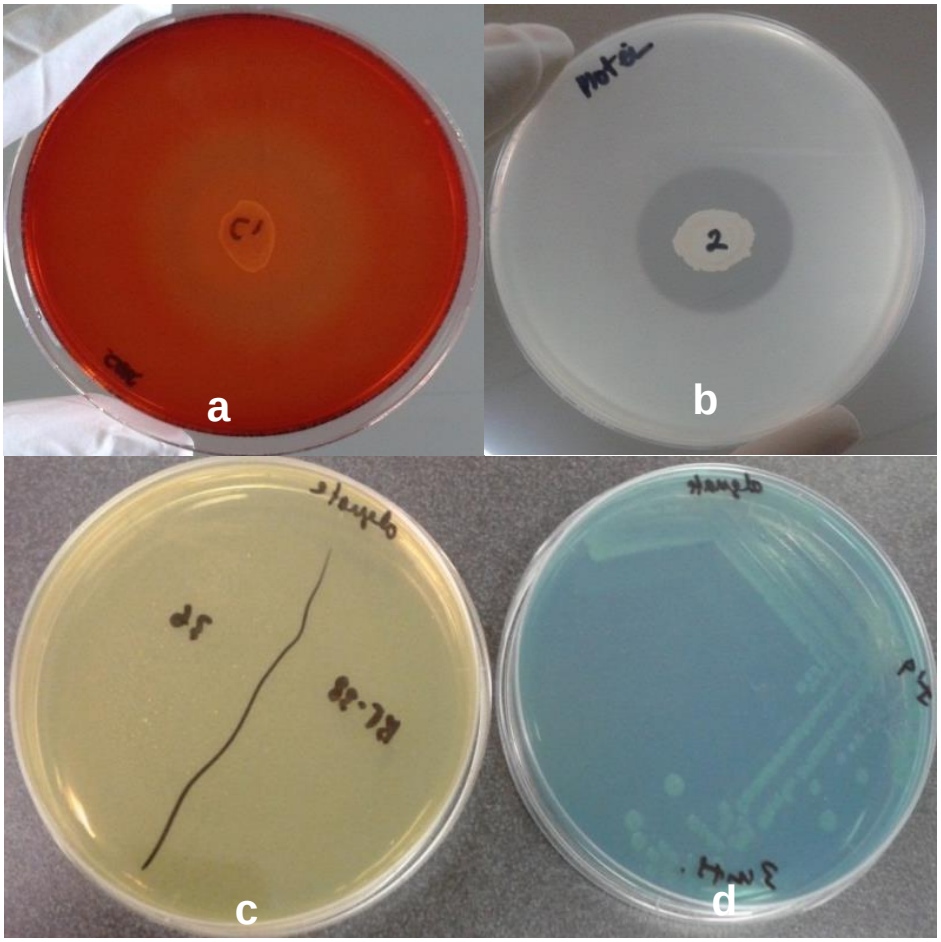


Figure 2. Bacterial strains producing digestive enzymes indicated by the formation of clearance zones around the bacterial colonies, (a) cellulase activity or (b) protease activity, (c & d) colour conversion from yellow to blue on alginate agar plates indicating alginate-lyase activity.

Phenotypically, 19 isolates were identified as Gram-positive and 5 isolates Gram negative. Additionally, most isolates produced catalase with a short or long bacilli cell shape (Table 7). Among these 50 % (11/24) seemed to lack oxidase, and the rest produced cytochrome oxidase.

Table 7. Phenotypic properties of enzyme-producing bacteria isolated from the GITs of abalone, sea bream and Australian salmon.

No	Bacterial isolate	Gram staining	Oxidase	Catalase	Cell Morphology and Arrangement	Source
1	Alg-1	+	-	+	Rod, single or pair	Ab
2	Alg-2	-	-	+	Coccus, single, chain	Ab
3	Alg-aw1	-	-	+	Rod, pair	Ab
4	Alg-aw2	-	+	+	Rod, pair	Ab
5	Alg- aw3	+	+	+	Short Rod, pair	Ab
6	Alg- aw5	+	-	+(w)	Rod, pair	Ab
7	C-1	+	-	+	rod, chain	Ab
8	C-2	-	-	+	Rod, pair	Ab
9	C-aw1	+	-	+	Rod, pair	Ab
10	C-aw2	+	+	+	Rod, chain	Ab
11	C-aw4	+	+	+(w)	Rod, pair	Ab
12	C-as4	-	+	+	Rod, chain	AuS
13	C-ab6	+	+	+	Rod, chain	Ab
14	LJ-24.1	+	-	+	Rod, chain	Ab
15	EJ-1	+	-	+	Rod, chain	Ab
16	Sb-1	+	+	+	Rod, pair	Sb
17	LJ-14.1	+	+	+	Rod, pair	Ab
18	GODR-13	+	+	+	Rod, chain	Ab
19	LJ-24.2	+	+	+	Rod, pair	Ab
20	LJ-20.1	+	+	+	Rod, single	Ab
21	GO-13	+	+	+	Rod, pair	Ab
22	Aw-1	+	+	+	Rod, pair	Ab
23	Lj-21	+	-	+	Rod, single	Ab
24	Bs	+	-	+	Rod, chain	Ab

Ab: abalone; AuS: Australian salmon, and Sb: seabream, W=weak

Fourteen representative isolates, based on colony appearance, morphology, and microscopic appearance, were genotypically identified by amplifying partial sequences of their 16S rRNA gene. The selected isolates were Alg-2, Lag-aw1, Alg-aw2, Alg-aw3, C1, C-2, C-aw2, C-aw4, C-as4, GO-13, Lj-20.1, Lj-21, Bs, and Lj-24.1. The results indicated that these isolates belonged to 9 genera and showed high similarities to 13 different species, Table 8.

Table 8. The identity of enzyme-producing bacteria isolated from abalone, sea bream and Australian salmon based on the sequence of partial 16S rRNA genes.

Isolates	The closest sequence in database	DNA similarity (%)	Accession number
Alginate lyase			
Alg-2	*Shigella sonnei*	99	NR_104826
Alg-aw1	*Enterobacter ludwigii*	99	NR_042349
Alg-aw2	*Achromobacter* sp.	99	NR_116198
Alg-aw3	*Achromobacter spanius*	99	NR_025686
Cellulase-producing strains			
C-1	*Bacillus toyonensis*	99	NR_121761
C-2	*Serratia quinivorans*	97	NR_037112
C-aw2	*Stenotrophomonas maltophilia*	94	NR_112030
C-as4	*Pseudomonas azotoformans*	99	NR_113600
C-aw4	*Shewanella baltica*	99	NR_025267
Protease-producing bacteria			
GO-13	*Bacillus cereus*	100	NR_074540
LJ-20.1	*B. toyonensis*	99	NR_121761
Lj-21	*Bacillus amyloliquefaciens* subsp. *plantarum*	99	NR_075005
Bs	*Bacillus methylotrophicus*	98	NR_116240
LJ-24.1	*Bacillus mycoides*	99	NR_113990

Acc.No; is the Accession number of the closest known organism in GenBank database.

A dendrogram based on partial sequencing of 16S rRNA gene GenBank data-base reference sequence was constructed using Geneious software, Figure 3. The dendogram analysis revealed a close relatedness between the bacterial isolates and the type strains. According to the classification scheme, the 14 bacterial isolates belonged to 8 genera and referred to 13 species.

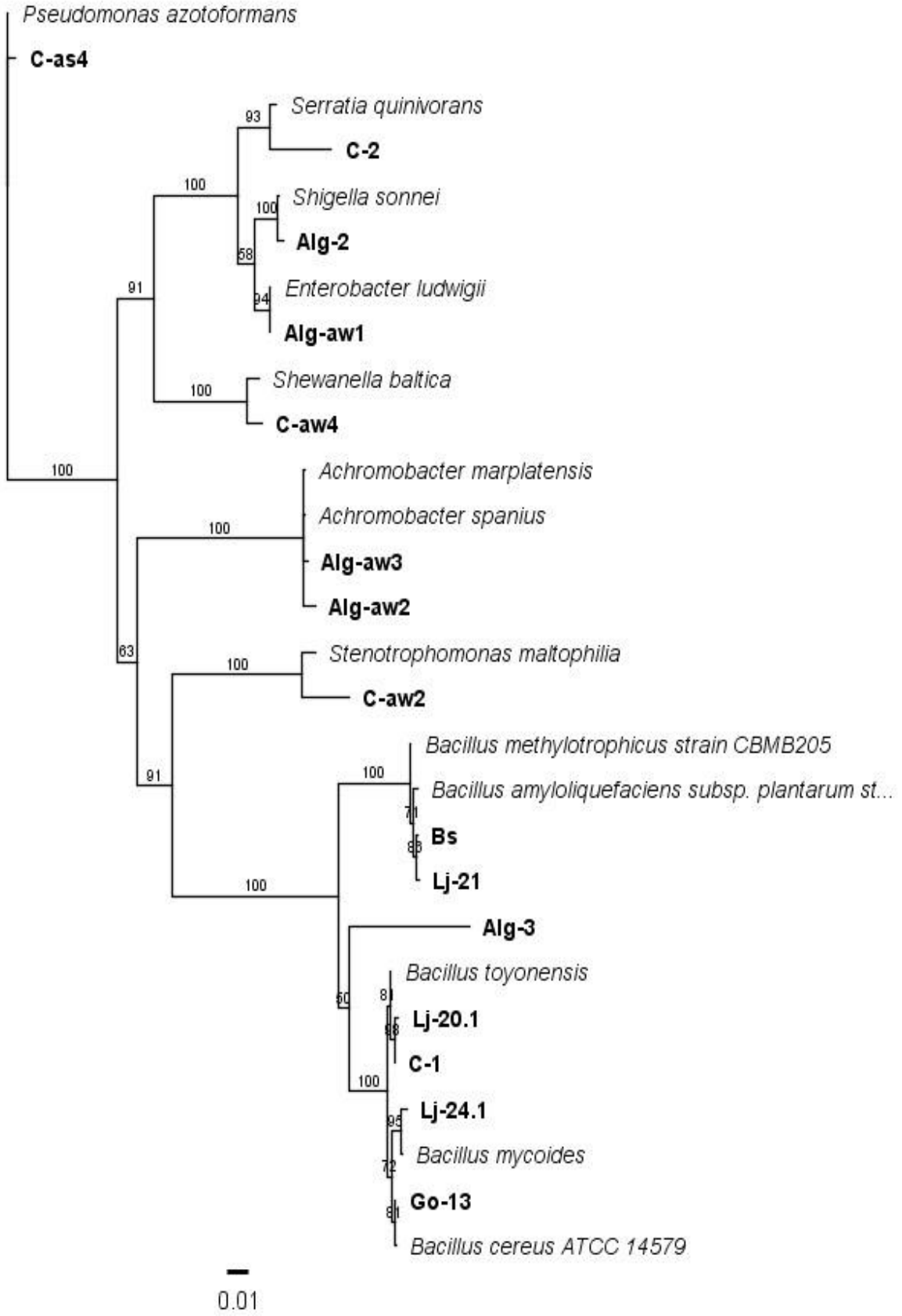


Figure 3. Dendogram showing phylogenetic relationships of representative enzyme-producing bacteria isolated from the GITs of teleosts and molluscs.

2.3.2. LAB

A total of 206 LAB were isolated from the GITs of all animal samples using both direct spreading and enrichment culture methods. These isolates consisted of: 20 LAB from Atlantic salmon, 91 LAB from juvenile and adult hybrid abalone, 52 LAB were isolated from sea bream, 8 isolates from trevally, 7 LAB from Australian salmon, 17 LAB from blue mussels, 4 LAB from jack mackerel, 2 LAB from long fin pike, 3 LAB from gurnard perch and 2 LAB from tiger flathead. The isolation of LAB using direct spreading method could be obtained only from GITs of Atlantic salmon and wild seabream. The average number of LAB isolated from Atlantic salmon was 1.10×10^2 CFU g^{-1}, and 1.65×10^2 CFU g^{-1} from GITs of wild seabream. No LAB were isolated from either brown trout, mullet, or the early juvenile of hybrid abalone

All isolates were Gram-positive, either cocci or short or long bacilli, lacked catalase and oxidase, and were able to ferment glucose. 62 representative isolates were selected and subjected to 16S rRNA gene (~800 bp) sequence analyses. The result revealed that these bacterial isolates belonged to seven genera and 23 LAB species, Table 9. Several LAB species appeared to be very specific, such as *Lactobacillus plantarum* which was only isolated from the GITs of blue mussel, *Weicella ceti* from GITs of seabream, *Leuconostoc mensenteroides* from GITs of hybrid abalone and *Lactobacillus farraginis* from GITs of Atlantic salmon. Some species were detected from several animals such *Carnobacterium divergens* from the GIT of seabream, Australian salmon, and hybrid abalone.

Table 9. The identity of LAB isolated from GITs of teleosts and molluscs

No	Isolate ID	The closest sequence in database	DNA similarity (%)	Accession number	Hosts
1	MA002	*Enterococcus faecium*	99	NR_113904	JA
2	MA033	*Enterococcus durans*	100	NR_113257	JA
3	MA034	*Lactobacillus curvatus*	99	NR_113334	JA
4	MA035	*Pediococcus acidilactici*	99	NR_042057	JA
5	MA037	*Enterococcus lactis*	99	NR_117562	JA
6	MA041	*E. durans*	99	NR_113257	JA
7	MA044	*Pediococcus pentosaceus*	99	NR_042058	JA
8	MA048	*Pediococcus lolii*	99	NR_042058	JA
9	MA056	*E. lactis*	99	NR_117562	JA
10	MA057	*E. faecium*	99	NR_113904	AA
11	MA059	*Lactobacillus sakei*	100	NR_113821	AA
12	MA064	*Leuconostoc mesenteroides*	99	NR_074957	AA
13	MA066	*Lactobacillus sakei*	99	NR_113821	AA
14	MA069	*E. faecium*	98	NR_113904	AA
15	MA070	*Leuc. mesenteroides*	99	NR_074957	AA
16	MA074	*Enterococcus maladoratus*	100	NR_114453	AA
17	MA075	*E. maladoratus*	100	NR_114453	AA
18	MA080	*Lb. sakei*	100	NR_113821	AA
19	MA081	*Lb. sakei*	99	NR_113821	AA
20	MA084	*E. lactis*	99	NR_117562	AA
21	MA087	*Lactococcus formosensis*	99	NR_114366	AA
22	MA090	*Carnobacterium divergens*	99	NR_113798	Sb
23	MA091	*Lactococcus garviae*	99	NR_113268	Sb
24	MA093	*Lact.garviae*	98	NR_113268	Sb
25	MA107	*Carnobacterium gallinarum*	99	NR_042093	Sb
26	MA108	*C. divergens*	99	NR_113798	Sb
27	MA109	*Enterococcus thailandicus*	100	NR_114015	Sb
28	MA112	*Lact. garviae*	100	NR_113268	Sb
29	MA118	*Lact. garviae*	100	NR_113268	Sb
30	MA120	*E. thailandicus*	99	NR_114015	Sb
31	MA122	*Enterococcus gilvus*	99	NR_113932	Sb
32	MA125	*Weisella* ceti	99	NR_119039	Sb
33	MA133	*W. ceti*	99	NR_119039	Sb
34	MA135	*Lactobacillus ginsenosidimutans*	95	NR_132607	Sb
35	MA142	*Lb. sakei*	100	NR_113821	Tr
36	MA147	*E. gilvus*	99	NR_113932	Tr
37	MA150	*Lactobacillus farraginis*	99	NR_041467	AtS
38	MA153	*Lb. farraginis*	99	NR_041467	AtS
39	MA156	*Pediococcus lolii*	99	NR_041640	AtS
40	MA160	*P. acidilactici*	99	NR_042057	AtS

41	MA161	*Lactobacillus pobuzihii*	99	NR_112694	AtS
42	MA163	*P. acidilactici*	99	NR_042057	AtS
44	MA164	*P. acidilactici*	99	NR_042057	AtS
45	MA169	*P. pentosaceus*	99	NR_042058	AtS
46	MA173	*E. gilvus*	99	NR_113932	Jm
47	MA174	*E. faecium*	99	NR_113904	Lfp
48	MA180	*Lact. garviae*	99	NR_113268	TFH
49	MA181	*Lactobacillus plantarum*	99	NR_104573	Ms
50	MA183	*Lb. sakei*	99	NR_113821	Ms
51	MA185	*Lactococcus lactis* subsp. *hordinae*	98	NR_113958	Ms
52	MA187	*Enterococcus faecalis*	99	NR_113901	Ms
53	MA188	*E. lactis*	99	NR_117562	Ms
54	MA189	*Lact. lactis* subsp. *hordinae*	99	NR_113958	Ms
55	MA195	*E. durans*	99	NR_113257	Ms
56	MA196	*E. lactis*	99	NR_117562	Ms
57	MA198	*E. faecalis*	99	NR_113901	JA
58	MA199	*P. acidilactici*	99	NR_042057	JA
59	MA200	*C. gallinarum*	98	NR_042093	AuS
60	MA204	*C. gallinarum*	98	NR_042093	AuS
61	MA205	*C. gallinarum*	99	NR_042093	AuS
62	MA206	*C. divergens*	99	NR_113798	AuS

Sb: seabream, AtS: Atlantic salmon, AuS: Australian salmon, JA: juvenile abalone, AA: adult abalone, Tr: trevally, Bm: blue mussel, Lfp: long fin pike, Jm: jack mackerel, Gp: gurnard perch. GenBank Acc No is the closest known organism.

The bacterial phylogenetic tree was constructed based on the partial 16S rRNA gene sequences of representative isolates. In general, the results showed that the representative isolates exhibited 84.8% sequence similarity and grouped into three main clusters, Figure 4. Cluster I was composed of two genera and seven species which were *Lb. ginsenosidimutans, Lb. plantarum, Lb. sakei, Lb. farraginis, Lb. curvatus, P. pentosaceus* and *P. acidilactici*. Cluster II consisted of two genera or two species which was *W. ceti* and *Leuc. mesenteroides*. The last cluster (III) consisted of four genera and 13 species which were *Lact. formosensis, Lact. garvieae, Lact. lactis* subsp. *hordinae* and *Lb. pobuzihii, C. divergens. C. gallinarum, E. faecalis, E. malodoratus, E. gilvus, E. durans, E. thailandicus, E. lactis,* and *E. faecium,* Figure 4.

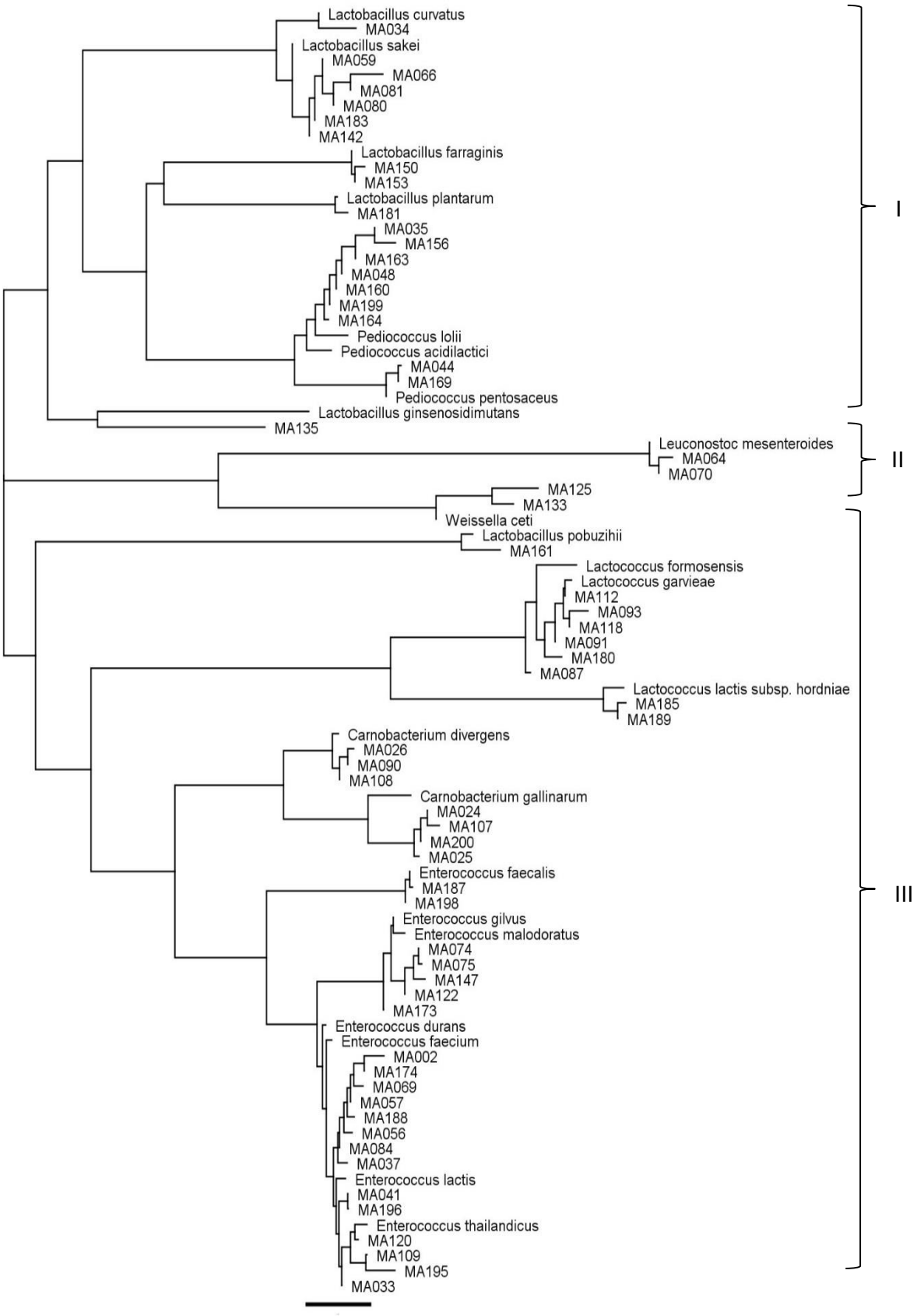


Figure 4. Phylogenetic relationships of representative LAB isolated from GITs of teleosts and molluscs based on partial 16S rRNA gene sequences. The phylogenetic tree was constructed by using geneious software with the neighbour-joining method.

2.4. Discussion

2.4.1. Isolation of intestinal microflora.

This study isolated 24 enzyme-producing bacteria and 206 LAB from the GITs of teleosts and molluscs using either direct spreading technique and an enrichment culture-dependent method. 76 representative isolates were selected for genotypic identification, and showed high similarity to 33 bacterial species, Table 8 and 9. The diversity of bacterial species might be underestimated due to the interference of several factors such as the isolation technique, culture media and environmental conditions used for the incubation (Ellis et al., 2003).

Isolation of targeted bacteria using the enrichment culture method appears to be too selective, since the author frequently observed only a single isolate from one sample, especially in LAB isolation. This result might relate to the capacity of LAB members to produce antimicrobial compounds (Verschuere et al., 2000a). Other LAB isolates in the GITs could be outcompeted by a strain exhibiting the strongest antimicrobial compounds. Furthermore, some members of LAB or enzyme-producing bacteria might require different nutrient sources (Ringo and Gatesoupe, 1998; LeBlanc et al., 2011). However, this present study relied mostly on MRS, a commercial medium which generally support the growth of 4 LAB genera: *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Leuconostoc* (De Man et al., 1960). On the other hand, the isolation of enzyme-producing bacteria used a simple modified medium, which might not support the growth of wider groups of enzyme-producing bacteria. In addition, the incubation temperature used in this present study was ~21 ^{o}C; which is different from the field temperature of 16 ^{o}C in which some of animal samples such as abalone were collected. All these factors might influence the number of LAB and enzyme-producing bacteria detected from the GITs of animal samples.

Several studies suggested to use more advanced techniques to give a more precise picture of intestinal microflora, such as polymerase chain reaction-denaturing gradient gel electrophoresis (Muyzer and Smalla, 1998) or other culture-independent metagenomic approaches (Nakamura et al., 2016). However, as the main concern of this study is not only detecting the presence of LAB and enzyme-producing bacteria but also investigating their potential capacity as aquaculture probionts, the use of the culture-dependent technique is considered to be legitimate.

2.4.2. Enzyme-producing bacteria

Digestive enzymes, such as protease, cellulase, amylase, and lipase, are required to break down feed particles into absorbable nutrients in the intestinal tract of cultivated animals (Ray et al., 2012). Besides these enzymes, different animals require additional types of digestive enzymes. For instance, abalone, which feed on macroalgae, require more alginate lyase, agarase and carraginase to digest the macroalgae (Erasmus et al., 1997). These enzymes can be produced by the animal hosts (endogenous enzymes) or by other exogenous sources such as intestinal bacteria. Some cultured animals lack of ability to produce certain enzymes. As consequence, the interest in external sources of digestive enzymes especially intestinal bacteria has been growing due to their potential contribution to the feed digestion and growth of cultivated animals. This present study isolated 24 enzyme-producing bacteria from the GITs of abalone, seabream, and Australian salmon: 11 protease-producing bacteria, 7 cellulase-producing bacteria and 6 alginate lyase-producing bacteria.

2.4.2.1.Protease-producing bacteria

The amount of protein deposition as tissue determines the growth of cultured abalone. Protein deposition is dependent on the protein content of feed and its digestibility by abalone, which is influenced by many factors especially the availability and activity of protease in digestive tract of abalone (Alnahdi, 2012). This present study isolated 11 indigenous bacteria which have the capacity to produce protease. All isolates appeared to be members of genus *Bacillus,* indicated by phenotypic studies (Table 5) and 16S rRNA gene sequences of 5 representative isolates (Table 6). These results are quite similar to previous studies in which many *Bacillus* species have been reported to produce protease, including *B. cereus* (Esakkiraj et al., 2009), *Bacillus circulans* (Bandyopadhyay and Das Mohapatra, 2009), *B. subtilis* (Ravishankar, 2012; Zokaeifar et al., 2012), *B. amyloliquefaciens* subsp. *plantarum* (Sai-Ut et al., 2013), *Bacillus licheniforemis* (Suganthi et al., 2013), *B. toyonensis* (Okaiyeto et al., 2015), and *Bacillus coagulans* (Gupta et al., 2016).

Three species isolated in this present study (*B. amyloliquefaciens, B. toyonensis,* and *B. cereus*) have been frequently reported as beneficial bacteria. *B. amyloliquefaciens* has been sold commercially as growth and health-promoting agents in aquatic animals (Ecobiol Aqua, Norel Animal Production, Attaka industrial zone-Suez Gulf, Egypt). Supplementation of the species at concentration of 1.0×10^{10} CFU mL^{-1} improve not only the growth of Nile tilapia (Reda and Selim, 2015), but also enhanced the nonspecific immune system (Selim and Reda, 2015). In addition, *B. cereus* isolated from the intestine of *Mugil cehpahlus* have been reported to produce protease and showed ability to digest several protein sources such as raw fish meat, defatted fish meat, and alkaline hydrolysate (Esakkiraj et al., 2009). Kurniasih et al. (2013) reported the addition of *B. cereus* into formulated pellet improved protein digestibility and growth of Tilapia. However, in this study, 2 species (*B.*

methylotrophicus, and *B. mycoides*) appear to be first reports of isolation from GITs of aquatic species.

These bacteria may contribute to protein utilization and growth rates of cultivated species, as previously confirmed in several aquatic animals: improving digestibility and retention of proteins in carp (Shi et al., 2016), Nile tilapia (Kurniasih et al., 2013), shrimp (Roumanas, 2010), and abalone (Hadi et al., 2014). Therefore, *in vivo* studies on these protease-producing bacteria need to be further investigated.

2.4.2.2. Alginate lyase-producing bacteria

Alginate lyase is an enzyme which can degrade alginate, one of major components in seaweed, through β-elimination of the glycosidic bond to yield various oligosaccharides (Michaud et al., 2003). This enzyme is very important for abalone which naturally feed on seaweed (Erasmus et al., 1997; Hadi et al., 2014). In this present study, a total of 6 bacteria exhibiting alginate-lyase activity were isolated from the GITs of hybrid abalone. Previously, alginate lyase-producing bacteria were reported from a wide range of environmental sources, including seaweeds (Kim et al., 2009), sea mud (Iwamoto et al., 2001) and also the GIT of abalone (Erasmus et al., 1997). Four representative isolates of these alginate lyase-producing bacteria were identified as *E. ludwigii, S. sonnei, A. marplatensis* and *A. spanius.* None of these species have previously been reported with alginate-lyase activity. Several bacteria with alginate lyase activity are *Pseudomononas* strain C4 (Erasmus et al., 1997), *Streptomyce*s sp. ALG-5 (Kim et al., 2009), *Sphingomonas* sp. (Miyake et al., 2003), *Alteromonas* sp. (Iwamoto et al., 2001) and *Vibrio* sp. (Tseng et al., 1992; Hadi et al., 2014).

A number of studies have confirmed that supplementation of alginate lyase-producing bacteria enhanced macroalgal digestion and improved the abalone growth (ten Doeschate and Coyne, 2008; Hadi et al., 2014). Acknowledging the beneficial contribution

of the isolated bacteria, screening for other probiotic parameters such as quantification of enzyme activity and their capacity to colonize the GIT of abalone need to be further studied.

2.4.2.3. Cellulase-producing bacteria

Cellulose is one of the main components of aquatic plants and so is considered to be the most abundant organic material in the world (Li et al., 2008; Ambas et al., 2015). Due to its β-1, 4-glycosidic bonds, cellulose is difficult to degrade by cultured animals. However, some studies indicated that cellulose could be degraded by certain strains of bacteria through the production of an enzyme called cellulase (Davies, 1965; Gupta et al., 2012; Liu et al., 2014). Cellulase is an enzyme which can hydrolyse the β-1, 4-glycosidic bonds in cellulose into simpler molecular forms such as cellobiose, cellotriose and other oligosaccharides and eventually into glucose to be utilized by their host, Figure 5 (Li et al., 2008; Rathnan et al., 2013).

In this present study, seven bacterial strains with the capacity to excrete cellulase were isolated from the GITs of Australian salmon and hybrid abalone. Based on 16S rRNA gene sequences, these isolates were identified as *B. toyonensis*, *P. azotoformans*, *S. quinivorans, S. maltophilia*. and *S. baltica*. Previously, cellulase-producing bacteria have also been reported from wood (Paudel and Qin, 2015), soil (Chang et al., 2014), and the intestines of aquatic species (Saha et al., 2006; Ray et al., 2010; Peixoto et al., 2011). Some studies indicated that cellulase-producing bacteria were commonly isolated from the GITs of herbivorous species including grass carp and tilapia (Saha et al., 2006). This is in agreement with the result of this study in which 6 out of 7 cellulase-producing bacteria were isolated from the GIT of abalone, which naturally feed on micro and macroalgae. Whether these cellulose-degrading bacteria can contribute to feed digestion of cultivated species needs to be further investigated.

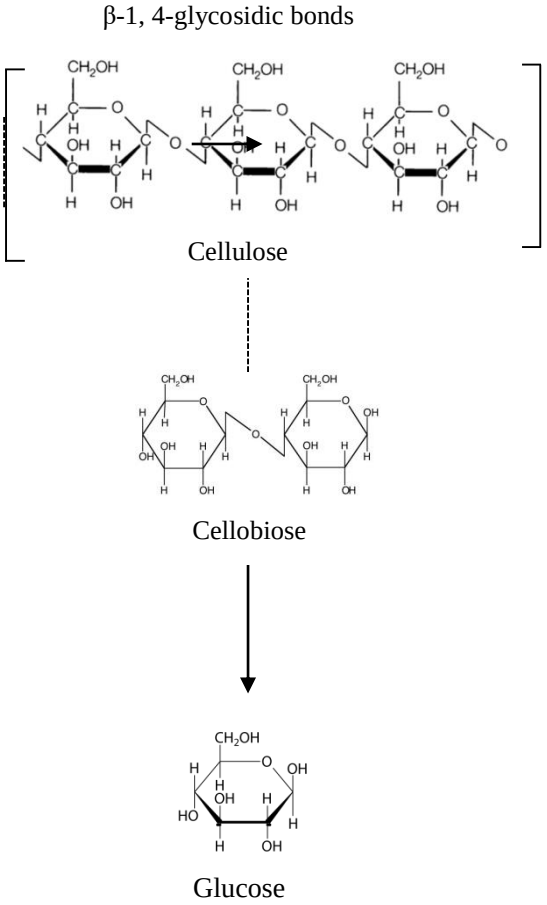


Figure 5. Degradation of cellulose by cellulase into glucose units

No bacteria which produce amylase or lipase could be isolated from this present study. This result was in contradictory with previous studies (Erasmus et al., 1997; Zhao et al., 2012; Hadi et al., 2014). Zhao et al. (2012) for instance, reported that lipase and amylase-producing bacteria could be isolated from GIT of early and juvenile abalone, *H. diversicolor*. Even, the lipase-producing bacteria were found to be dominant in early juvenile (3 mm) and late juvenile (20 mm) which feed on diatoms. The difference species and rearing condition might affect the results. In addition, some factors such as temperature, pH, culture medium, aerobic condition during incubation may also affect the number and diversity of enzyme-producing bacteria being recovered (Shi et al., 2015).

This present study used 1:2-5 diluents, which is suggested in previous studies. However, the result obtained from direct spreading method only LAB from Atlantic salmon and seabream.

2.4.3. LAB

Several studies reported that LAB are not numerically dominant in the intestinal tracts of aquatic species. However, they are regarded as part of normal intestinal microflora in healthy animals (Verschuere et al., 2000a), which give many beneficial effects to their host. LAB have been documented to produce: antimicrobial compounds against various bacterial pathogens (Cintas et al., 1997; Ghanbari et al., 2013), vitamins (Hugenschmidt et al., 2010; LeBlanc et al., 2011; Masuda et al., 2012; LeBlanc et al., 2013), fatty acids (Iehata et al., 2009; 2010; Pessione et al., 2015), and digestive enzymes (Askarian et al., 2011).

LAB are well known for their capacity to inhabit under wide range of habitats. They were isolated from plants (Hwanhlem et al., 2014), soil (Yanagida et al., 2006), poultry (Maldonado et al., 2012; Sakaridis et al., 2014; Shazali et al., 2014), and humans (Rubio et al., 2014). In addition, LAB have been reported from the GITs of aquatic animals, including shrimps (Maeda et al., 2014), Nile tilapia (Lara-Flores and Olvera-Novoa, 2013), African catfish (Hamid et al., 2012), seabass (Bourouni et al., 2012), trout (Perez-Sanchez et al., 2011), sturgeon (Askarian et al., 2011), and abalone (Hagi and Hoshino, 2009). This present study confirmed that LAB inhabit GITs of Atlantic salmon, Australian salmon, abalone, seabream, trevally, mussel, long fin pike, jack mackerel, and gurnard perch, collected different environmental conditions (recirculating aquaculture systems, flow–through aquaculture systems, estuarine and marine water).

In terms of taxa, this present study detected 7 LAB genera: *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus,* and *Weisella*, which

are quite similar to the LAB genera previously detected from the GIT of Atlantic salmon in a Tasmanian fish farm (Zarkasi et al., 2014). The only difference is that this study frequently isolated *Pediococcus* from the GITs of Atlantic salmon, but *Pediococcus* was not reported by Zarkasi et al. (2014). On the other hand, *Streptococcus*, which was detected in Zarkasi's study, was undetected in this present study. These differences might be related to the difference in the number of fish samples, the culture system (a recirculating aquaculture system vs open sea cages) and the detection method (culture-dependent vs molecular methods) between this study and Zarkasi's study.

The phylogenetic tree which was constructed based on the partial 16S rRNA gene sequences showed that the isolated LAB were grouped into three main clusters. Cluster I consisted of 2 genera and 7 species, cluster II (2 genera and 2 species), and cluster III consisted of four genera and 13 species, Figure 4. In general, the clusters were very similar to what has been reported by (Makarova et al., 2006) who built a phylogenetic tree based on genome of lactic acid bacteria. For instance, *Lactobacillus* was closer to *Pediococcus*, and for the present study they were put in the same cluster.

2.4.3.1. Diversity of LAB

This present study showed that GITs of animals collected from the closed rearing environment tend to host fewer bacterial taxa than animals from either semi-closed system or open environments. As presented in Table 10, only two LAB genera (three species) were isolated from the GIT of Atlantic salmon which were reared in the closed rearing system. This number was much lower compared to the LAB genera or species isolated from animals reared in the flow-through aquaculture system (6 genera/14 species) or those animals inhabiting estuarine and marine environments (6 genera/16 species). This present result was in agreement with a previous study by Bucio et al. (2006) in which LAB associated with fish reared in a closed recirculating aquaculture system appeared to be fewer and less diverse

compared to fish collected from a river. The same conclusion was reported by Ringø and Strøm (1994), in which population level of LAB in the gastrointestinal tract of wild fish tended to be higher than cultured fish.

No LAB were isolated from either mullet or brown trout cultivated in the RAS system in this present study. The isolation techniques might influence the obtained results. However, 2 different techniques were used which were the direct spreading with a dilution factor (1:2-5) and the enrichment culture method, and still no LAB could be obtained from either mullet or brown trout. This suggests that the method was appropriate, thus the source of the fish appears to be a factor. Similarly, Bucio et al., (2006) reported that no LAB could be detected from the GITs of African catfish and tilapia reared in the same type of aquaculture system, a RAS. In contrast, freshwater fish such as carp and catfish (Hagi et al., 2004), trout (Perez-Sanchez et al., 2011), catfish (Hamid et al., 2012), Beluga and Persian sturgeon (Askarian et al., 2009) collected from open-water systems had been reported to be associated with numerous members of LAB.

Table 10. The composition of culturable LAB colonizing the GITs of teleosts and molluscs collected from different habitats.

LAB isolates	**Closed environment[a]**	**Semi-closed environment[b]**	**Open environment[c]**
Total of LAB isolates	20	91	95
Genera	2	6	6
Species	3	14	16

a = recirculating aquaculture system, b = flow-through rearing system, and c = estuarine and marine water.

There are various factors which have been described to affect the number and diversity of LAB associated with GIT of aquatic animals, including rearing system, diets,

and salinity affected (Ringo and Olsen, 1999; Tanaka et al., 2003). Fish living in more open system (wild environments), for instance, generally feed on more variety of diets such as phytoplankton and zooplankton, bacteria, plants, and other small aquatic animals. Meanwhile, fish reared in a closed-aquaculture system feed mostly on artificial diet such as manufacture pellet. Rearing water in closed-aquaculture system are generally treated before entering the aquaculture system, such as ultraviolet, ozonization, or filtered through series of filter, 1 µm, 0.5 µm and 0.22 µm to reduce bacterial loads to the system. Thus, source of bacteria is mostly from feed, whilethe bacteria may come also from feed and rearing water in wild fish. Ringø and Strøm (1994) concluded that salinity also affects the diversity and number of LAB in GIT of fish. For instance, percentage of *Lactobacillus* sp. in Arctic charr, *Salvelinus alpinus*, reared in fresh water was larger than marine water.

2.4.3.2. Dominant LAB

Enterococcus appeared to be the most frequently genus isolated from the GITs of the teleosts and molluscs collected from both semi-closed and open environments, Figure 6. This result is in agreement with a study conducted by Bourouni et al. (2012) on the intestinal tract of European seabass (*Dicentrarchus labrax*). However, other studies also reported that different aquatic species and rearing environments might be dominated by different genera of LAB. *Pediococcus* was the most frequently observed cultivable LAB from the GITs of Atlantic salmon, Figure 6a. In contrast, *Lactococcus* was reported to be dominant in Atlantic salmon reared in seawater (2014), and in Persian sturgeon (Soltani et al., 2013). In addition, *Lactobacillus* was dominant in the intestinal tract of kuruma shrimp (*Marsupenaeus japonicas*) (Maeda et al., 2014) and several freshwater fish (Boulares et al., 2011). These results may indicate that various factors, including rearing water and species, contribute in shaping the dominant LAB colonizing the intestinal tract of aquatic species.

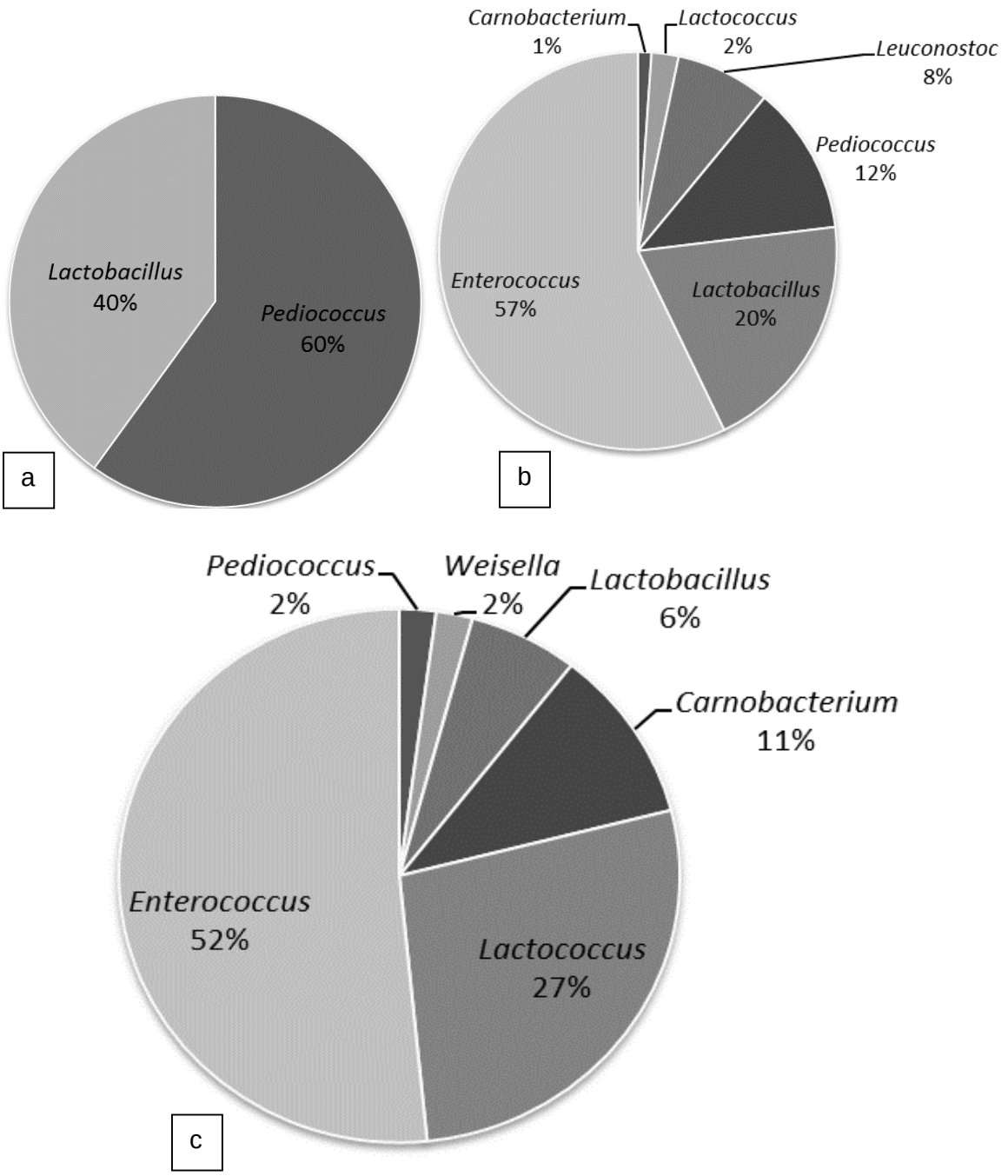


Figure 6. The proportion of cultivable LAB genera which was isolated from the GITs of teleosts and molluscs collected from different environmental conditions in Tasmania. a = closed rearing system (Atlantic salmon, n=20), b = semi-closed rearing system (abalone, n=91), and c = open water system (seabream, mussels and marine fish, n=95).

2.5. Conclusion

A total of 230 bacteria were isolated from the GITs of 155 teleosts and molluscs using either a direct plating culture method or an enrichment-dependent culture method. Twenty-four bacteria isolates displayed the capacity to utilize either one of these substrates: casein, sodium alginate and carboxymethyl cellulose, which may indicate that these bacteria are able to synthesize either protease, alginate lyase or cellulase. The other 206 isolates were members of LAB. According to their 16S rRNA gene sequences, these bacteria belonged to 16 genera and 36 species. Screening and characterization of all bacterial isolates for the production of either extracellular digestive enzymes or bacteriocin-like inhibitory substances against bacterial pathogens were studied in chapters 3 and 4.

Chapter 3 : Screening and Characterization of Enzyme - Producing Bacteria for Probiotic Candidates

3.1. Introduction

Various strains of bacteria have been documented to enhance feed digestion and improve the growth of many economically important aquatic species such as Indian shrimp, *Fenneropemaeus indicus,* (Ziaei-Nejad et al., 2006; Wang, 2007; Zokaeifar et al., 2012; Nimrat et al., 2013), grouper, *Epinephelus coioides* (Sun et al., 2011; Yan et al., 2016), carp, *cyprinus carpio,* (Wang and Xu, 2006), catfish, *Clarias gariepinus,* (Al-Dohail et al., 2009) and Nile tilapia, *Oreochromis niloticus,* (Ridha and Azad, 2012; Lara-Flores and Olvera-Novoa, 2013; Reda and Selim, 2015). Several authors reported that these bacteria contributed to their host by secreting important digestive enzymes such as protease, amylase, lipase, cellulase or alginate lyase (Erasmus et al., 1997; Ray et al., 2012; Hadi et al., 2014). Thus, many strains of enzyme-producing bacteria have been developed commercially as feed supplements for aquaculture species, generally known as probionts. However, some studies argued that the use of commercial probionts is often less effective because they may have a low ability to live and colonize the intestinal tract of their new hosts. Therefore, more screening parameters are needed to improve selection of probiotic candidates for aquaculture species.

There are several criteria to select probiotic candidates for aquaculture species including: the capacity to produce digestive enzymes (Balcazar et al., 2006; Kesarcodi-Watson et al., 2008; Martinez Cruz et al., 2012), high tolerance to rearing water (Gatesoupe, 1999; Balcazar et al., 2006), good capacity to reach and colonize the GITs of animal hosts (Verschuere et al., 2000a; Geraylou et al., 2014), and non-toxic to the cultured animals (Verschuere et al., 2000a). Due to these parameters, there was a general consensus that the best place to look for probiotic candidates was from the indigenous bacterial communities of aquatic animals (Fjellheim et al., 2010). Endogenous bacteria are presumed to be well adapted to the targeted ecological niche, therefore have greater chance of colonizing host

guts and conferring beneficial contribution (Fjellheim et al., 2010; Geraylou et al., 2014). Viability of probiotic candidates can regulate the activity of probiotic candidates for their animal host (Nayak, 2010b). Thus, these parameters should be investigated to select probiotic candidates for aquaculture species.

This study aimed at the screening of probiotic candidates from the endogenous bacteria of teleost and molluscs. Probiotic candidates were selected based on several criteria: the capacity to produce at least one of five digestive enzymes (protease, amylase, cellulase, alginate lyase and lipase), a capacity to tolerate GITs environmental conditions (stomach and intestine), and for their safety to cultivated animals, viability in rearing water and pellets, toxicity and susceptibility to antibiotics.

3.2. Materials and Methods

3.2.1. Tested bacteria

A total of 230 endogenous bacteria (from chapter 2) were used in this study (Table 11). These bacteria were individually subcultured in either MRS or MH broth. After 24-h incubation at room temperature, the broth culture was plated onto either MRS or MH agar. A fresh single colony of each tested bacterium was taken from the agar plate, and subcultured in 5 mL of either MRS or MH broth for further assays.

Table 11. Bacterial isolates used in this present study.

No	Isolate ID	Source	No	Isolate ID	Source	No	Isolate ID	Source	No	Isolate ID	Source	No	Isolate ID	Source
1	MA001	JA	47	MA047	JA	93	MA093	Sb	139	MA139	Sb	185	MA185	Bm
2	MA002	JA	48	MA048	JA	94	MA094	Sb	140	MA140	Sb	186	MA186	Bm
3	MA003	JA	49	MA049	JA	95	MA095	Sb	141	MA141	Sb	187	MA187	Bm
4	MA004	JA	50	MA050	JA	96	MA096	Sb	142	MA142	Trev	188	MA188	Bm
5	MA005	JA	51	MA051	JA	97	MA097	Sb	143	MA143	Trev	189	MA189	Bm
6	MA006	JA	52	MA052	JA	98	MA098	Sb	144	MA144	Trev	190	MA190	Bm
7	MA007	JA	53	MA053	JA	99	MA099	Sb	145	MA145	Trev	191	MA191	Bm
8	MA008	JA	54	MA054	JA	100	MA100	Sb	146	MA146	Trev	192	MA192	Bm
9	MA009	JA	55	MA055	JA	101	MA101	Sb	147	MA147	Trev	193	MA193	Bm
10	MA010	JA	56	MA056	JA	102	MA102	Sb	148	MA148	Trev	194	MA194	Bm
11	MA011	JA	57	MA057	GA	103	MA103	Sb	149	MA149	Trev	195	MA195	Bm
12	MA012	JA	58	MA058	GA	104	MA104	Sb	150	MA150	AtS	196	MA196	Bm
13	MA013	JA	59	MA059	GA	105	MA105	Sb	151	MA151	AtS	197	MA197	Bm
14	MA014	GA	60	MA060	GA	106	MA106	Sb	152	MA152	AtS	198	MA198	GA
15	MA015	GA	GA	MA061	GA	107	MA107	Sb	153	MA153	AtS	199	MA199	GA
16	MA016	GA	62	MA062	GA	108	MA108	Sb	154	MA154	AtS	200	MA200	AuS
17	MA017	GA	63	MA063	GA	109	MA109	Sb	155	MA155	AtS	201	MA201	AuS
18	MA018	GA	64	MA064	GA	110	MA110	Sb	156	MA156	AtS	202	MA202	AuS
19	MA019	GA	65	MA065	GA	111	MA111	Sb	157	MA157	AtS	203	MA203	AuS
20	MA020	GA	66	MA066	GA	112	MA112	Sb	158	MA158	AtS	204	MA204	AuS

21	MA021	GA	67	MA067	GA	113	MA113	Sb	159	MA159	AtS	205	MA205	AuS
22	MA022	GA	68	MA068	GA	114	MA114	Sb	160	MA160	AtS	206	MA206	AuS
23	MA023	GA	69	MA069	GA	115	MA115	Sb	161	MA161	AtS	207	Alg-1	AuS
24	MA024	GA	70	MA070	GA	116	MA116	Sb	162	MA162	AtS	208	Alg-2	AuS
25	MA025	GA	71	MA071	GA	117	MA117	Sb	163	MA163	AtS	209	Alg-aw1	GA
26	MA026	GA	72	MA072	GA	118	MA118	Sb	164	MA164	AtS	210	Alg-aw2	GA
27	MA027	GA	73	MA073	GA	119	MA119	Sb	165	MA165	AtS	211	Alg- aw3	GA
28	MA028	JA	74	MA074	GA	120	MA120	Sb	166	MA166	AtS	212	Alg- aw5	GA
29	MA029	JA	75	MA075	GA	121	MA121	Sb	167	MA167	AtS	213	C-1	GA
30	MA030	JA	76	MA076	GA	122	MA122	Sb	168	MA168	AtS	214	C-2	GA
31	MA031	JA	77	MA077	GA	123	MA123	Sb	169	MA169	AtS	215	C-aw1	GA
32	MA032	JA	78	MA078	GA	124	MA124	Sb	170	MA170	Jm	216	C-aw2	GA
33	MA033	JA	79	MA079	GA	125	MA125	Sb	171	MA171	Jm	217	C-aw4	GA
34	MA034	JA	80	MA080	GA	126	MA126	Sb	172	MA172	Jm	218	C-as4	AuS
35	MA035	JA	81	MA081	GA	127	MA127	Sb	173	MA173	Jm	219	C-ab6	Ab
36	MA036	JA	82	MA082	GA	128	MA128	Sb	174	MA174	Lfp	220	LJ-24.1	AuS
37	MA037	JA	83	MA083	GA	129	MA129	Sb	175	MA175	Lfp	221	EJ-1	JA
38	MA038	JA	84	MA084	GA	130	MA130	Sb	176	MA176	Gp	222	Sb-1	Sb
39	MA039	JA	85	MA085	GA	131	MA131	Sb	177	MA177	Gp	223	LJ-14.1	JA
40	MA040	JA	86	MA086	GA	132	MA132	Sb	178	MA178	Gp	224	GODR-13	Sb
41	MA041	JA	87	MA087	GA	133	MA133	Sb	179	MA179	Tfh	225	LJ-24.2	JA
42	MA042	JA	88	MA088	GA	134	MA134	Sb	180	MA180	Tfh	226	LJ-20.1	JA
43	MA043	JA	89	MA089	GA	135	MA135	Sb	181	MA181	Bm	227	GO-13	GA

44	MA044	JA	90	MA090	Sb	136	MA136	Sb	182	MA182	Bm	228	Aw-1	GA
45	MA045	JA	91	MA091	Sb	137	MA137	Sb	183	MA183	Bm	229	Lj-21	JA
46	MA046	JA	92	MA092	Sb	138	MA138	Sb	184	MA184	Bm	230	Bs	GA

Sb: seabream, AS: Atlantic salmon, AuS: Australian salmon, GA: Adult Abalone, JA: juvenile abalone, Tr: trevally, Bm: Blue mussel, Lfp: long fin pike, Jm: jack mackerel, Gp: gurnard perch.

3.2.2. Screening and quantification of enzymatic activity

3.2.2.1. Screening of enzyme production

Screening of the 230 bacteria for enzyme production was performed according to the protocol as described in chapter 2.

3.2.2.2. The enzyme activity

Protease: This assay was performed according to a modified protocol of Zhou et al. (2009). In brief, 10 µL of fresh bacterial culture was spotted onto a casein agar medium and incubated aerobically at room temperature for two days. Then, the proteolytic activity was confirmed by the formation of a clear zone around the colony. Quality of protease produced by each bacterial strain was measured by calculating the ratio between the diameter of the clearance zone and the diameter of the related bacterial colony (Taechapoempol et al., 2011). Each bacterial strain had 2 replicates.

Cellulase: Quantification of cellulase activity was investigated according to a modified protocol of Miller (1959). In brief, cellulase-producing strains were grown in nutrient broth supplemented with CMC 3 g L^{-1} for three days at room temperature, aerobically. Each bacterial strain had 3 replicates. Then the broth culture was harvested at early stationary phase by centrifuging at 4,000 x *g* for 15 min at 4 ^{o}C and the supernatant was harvested. One ml of the supernatant was mixed with 3,5-dinitrocalicylic acid (DNS) reagent (0.2 % phenol, 1% sodium hydroxide and 0.05 % sodium sulphite), and boiled for 5 min. The mixture was then diluted (1:10) with distilled water and its optical density was measured at optical density (OD) 540 nm of wavelength using an Infinite M200Pro spectrophotometer (TECAN). Several ranges of solution with different ranges of glucose concentration were made and treated the same way as the samples, as a control.

Alginate lyase: Fresh broth cultures of tested bacterium were plated onto alginate agar and incubated at room temperature (22 ^{o}C) for two days, aerobically. Colonies which produced a blue halo zone on the agar plates were considered to produce alginate lyase (Nakamura, 1987).

Quantification of alginate-lyase activity was determined by quantifying the amount of reducing sugar formed from sodium alginate according to Miller (1959), with slight modification. Briefly, the test bacteria were subcultured in the alginate broth medium and incubated at room temperature aerobically for three days. Cell-free supernatant was collected at early stationary phase by centrifugation at 4,193 x *g* for 15 min at 4 ^{o}C. Each bacterial strain had three replicates. Then, one mL of the culture supernatant was mixed with 1 mL of 3,5-DNS solution and boiled for 5 min. After cooling at room temperature, the absorbance of the mixed solution was measured at 540 nm after a 1:10 dilution with distilled water (results were expressed as mg glucose L^{-1}).

3.2.3. Viability in commercial pellets

Fresh broth cultures of each bacterium were adjusted to an OD value of 0.1-0.15 at 600 nm, and a 10-mL aliquot of each was sprayed individually onto 10 g of sterilized commercial pellets (ABFEED, MARIFEED) on sterilized aluminum foil. Subsequently, the commercial feed was air dried for 10 min at room temperature before being stored at 4 ^{o}C for one week. The viability of the added bacteria was monitored by taking 1 g of the bacteria-impregnated feed and diluting in 9 mL of 0.85 % saline solution. This mixture was then serially diluted, and each serial dilution was plated onto duplicate tryptic soya agar (TSA) plates every 24 h for one week.

3.2.4. Viability in rearing water

Fresh broth cultures of each enzyme-producing isolate were centrifuged, washed, and suspended into sterile 0.85 % NSS. The mixture was adjusted to an OD value of 0.1-0.15 at 600 nm, and 100 µL was inoculated into duplicate 10 mL sterilized seawater (32 ppt) aliquots, followed by 6 h incubation at room temperature (21.3±2.4 ºC). The viable cells of enzyme-producing bacteria were investigated by making two separate dilution series (10^{-1}-10^{-5}) made from each duplicate sample, at 0 h and after 6 h incubation. Then, each dilution had 2 agar plates (plated from 10^{-2} – 10^{-5}).

3.2.5. Viability in a simulated stomach juice (SSJ)

The ability of enzyme-producing bacteria to survive in the stomach environment was investigated by exposing them into a simulated stomach juice according to a protocol developed by Geraylou et al. (2014), with slight modification. In brief, fresh bacterial cultures were adjusted to an OD value of 0.1-0.15 at 600nm (1.0×10^5 CFU mL^{-1}), and 100 µL of each was incubated in duplicates of 10 mL simulated stomach juice (SSJ), which consisted of: pepsin at 3 mg mL^{-1} in 0.85 % NSS with pH 5.53, being the pH in the stomach of abalone (Harris et al., 1998). The mixtures were then incubated at 21.3±2.4 ºC aerobically for 3 h at static condition. Cell viability of the tested bacteria was measured by plating onto TSA plates at 0 h (initial cell numbers) and after 3 h.

3.2.6. Viability in a simulated intestinal juice (SIJ)

This assay was done according to a modified protocol of Geralylou et al. (2014). Briefly, tested bacteria grown in 5 mL of MRS broth overnight were centrifuged, washed, and resuspended in sterile 0.85 % NSS to a concentration of ± 1.0×10^7 CFU mL^{-1}. Thereafter, 50 µL of this suspension was inoculated into duplicates of 5 mL simulated intestinal juice with the following composition: 125 mM of NaCl (BDH Australia), 7 mM of KCl (Merch

Germany), 45 mM of $NaHCO_3$ (Merck Germany), 0.3 % bile salt (LP0056, Oxoid), 3 g L^{-1} of trypsin (0152159, Difco) and pH of 7.4. Then, the mix was incubated at 21.3±2.4 ºC aerobically. Cell viability was evaluated by making serial dilutions of the mixture and plating onto TSA at 0 h and after 4 h.

3.2.7. Toxicity assay

Feed preparation: 10 g of feed was placed on aluminum foil and autoclaved for sterilization. Afterward, the feed was sprayed with fresh broth culture of tested bacteria at concentration of ~1.0 x 10^9 CFU g^{-1}. The feed was stored at 4 ºC until further used. The bacteria impregnated feed was prepared once in three days.

Toxicity assay: Forty juvenile hybrid abalone (*H. rubra* vs *H. laevigata*) weighing 0.47 ± 0.12 g were divided into four experimental groups in a small-scale, *in vivo* experiment. Each group had two rearing tanks and each tank had five juvenile abalone. The abalone were fed with 1.5 % BW.day^{-1} with commercial pellets which were previously impregnated with each representative enzyme-producing bacterium (~1.0 x 10^9 CFU g^{-1}). The animals were reared in 10L filtered seawater (32 ppt) for 14 days, and the rearing water was replenished 100% every three days. Seawater was filtered through three series of different filters, 1; 0.45 and 0.2µm. During the experiment, abalone mortality was recorded daily, and dead animals were removed.

3.2.8. Susceptibility to antibiotics

Susceptibility of bacterial strains to several antibiotics including: chloramphenicol (10 µg/mL, 30 µg/mL), oxytetracycline (30 µg/mL), carbencillin (100µg/mL) novobiocin (5 µg/mL), ampicillin (10 µg/mL), and vancomycin (60 µg/mL) was assessed by a disc diffusion assay according to Perez-Sanchez *et al.* (2011). Briefly, one mL of fresh broth culture of each bacterial isolate (OD_{600}:0.1) was poured into MH agar (1.7 % agar) and air

dried for 15 min. at room temperature. Thereafter, the antibiotic disks were placed on the agar and incubated at 22 ^{o}C for 24 h. The diameters of the growth inhibition halos around the antibiotic disks were measured, and only those with diameter of more than 10 mm were considered as susceptible to the tested antibiotics.

3.2.9. Data analysis

The collected data for enzyme activity, viable cells recorded from survival experiments in seawater, feed matrix, SSJ, and SIJ were analysed using SPSS software version 22. A Paired Sample t-test or one-way analysis of variance (ANOVA) and Tukey post hoc test were used to determine significant difference between the sample means at $P<0.05$ after testing for normality and homogeneity of variance.

3.3. Results

3.3.1. Enzyme-producing bacteria

Out of the 230 screened indigenous bacteria, only 24 isolates were confirmed to produce at least one of these enzymes (protease, cellulase and alginate lyase). These enzyme-producing bacteria were composed of 11 isolates with protease activity, 7 isolates with cellulase activity and 6 isolates with alginate-lyase activity. None of these bacteria was a member of the LAB.

3.3.1.1. Protease

Among the positive strains, Lj21, which was identified as *B. amyloliquefaciens* subsp. *plantarum*, displayed the highest hydrolytic capacity (HC) on the casein-agar, with an HC of 3.3 followed by LJ-24.2 (3.2) and Sb-1 (3.1), Table 12.

Table 12. hydrolytic activity of eleven strains of proteinase-producing bacteria isolated from the GITs of teleosts and molluscs

No	Bacterial strains	Bacterial Species	Hydrolytic Capacity (HC)
1	LJ-24.1	*Bacillus mycoides*	+
2	EJ-1	Unknown	+
3	Sb-1	Unknown	+++
4	LJ-14.1	*B. toyonensis*	++
5	GODR-13	Unknown	+++
6	LJ-24.2	Unknown	+++
7	LJ-20.1	Unknown	+++
8	GO-13	*B. cereus*	++
9	Aw-1	Unknown	++
10	Lj-21	*B. amyloliquefaciens* subsp. *plantarum*	+++
11	Bs	*B. methylotophicus*	+++

HC is the ratio between diameter of clearance zone and bacterial colony. + (0-1.9), ++ (2-2.9), +++ (>3)

3.3.1.2. Cellulase

Quantification of cellulolytic activity indicated that there was a significant difference in the amount of reducing sugar among the seven bacterial strains, $F=86.37$, df 7,10, $P<0.01$. In general, the highest amount of reducing sugar (glucose) was displayed from a cell-free supernatant extracted from *S. maltophilia*. strain C-aw2, at 204.9 ± 0.5 mg L^{-1}. This amount was three times higher compared to what was detected from *B. toyonensis* strain C1, with the second highest amount of reducing sugar (66.8 ± 19.8 mg L^{-1}), Figure 7**.**

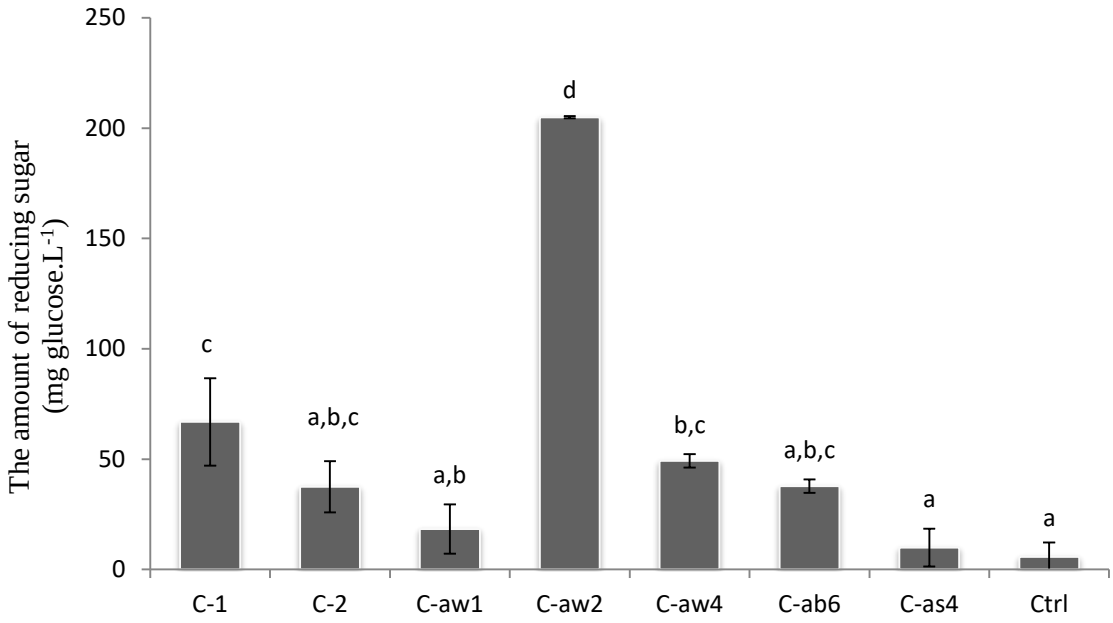


Figure 7. Cellulase activity of seven bacteria obtained from aquatic animals. Values are the average of glucose concentration with standard deviation from three replicates. Different letters indicate significant differences incellulase activity, $P<0.05$. Bacteria isoaltes; C1: *B. toyonensis*, C-aw2: *S. maltophilia*., C-aw4: *S. baltica*, C-as4, *P. azotoformans*, C-2, C-aw1, and C-ab6 were not identified.

3.3.1.3. Alginate lyase

This present study confirmed six bacterial strains with alginate-lyase activity, Figure 8. Based on quantification of alginate-lyase activity, there was a significant difference in the amount of reducing sugar in the cell free supernatant of these five bacterial strains, $F=19.4$, df 6, 12, $P<0.01$. Of these, strain Alg-aw1 which was identified as *E. ludwigii*, displayed the highest alginate-lyase activity indicated by the highest amount of reducing sugar, at 198.3 ± 7.2 mg L^{-1}.

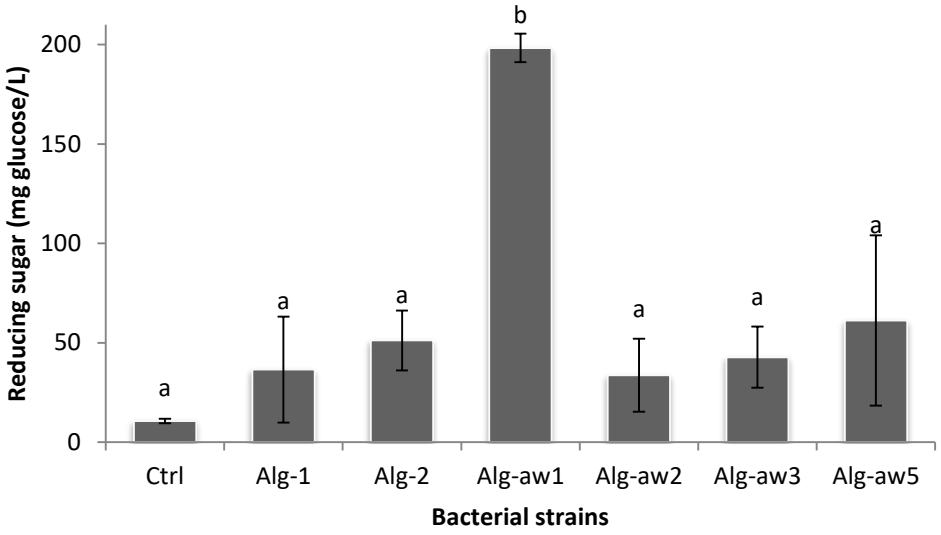


Figure 8. The amount of reducing sugar produced by six intestinal bacteria isolated from the GIT of aquatic species. Values are the average amount of reducing sugar with standard deviations of three replicates. Different letters indicate significant differences, P<0.05. Alg2: *S. sonnei*, Alg-aw1: *E. ludwigii*, Alg-aw2: A*chromobacter* sp., Alg-aw3: *A. spanius*, Alg-1, Alg-2, Alg-aw5 were not identified.

Three bacterial strains were selected to investigate their adaptability to different environmental conditions, including the survival rate in feed matrix stored in storage conditions, rearing seawater, simulated GIT conditions, toxicity, and susceptibility to antibiotics. The three bacterial isolates exhibiting the highest hydrolytic capacity were *B. amyloliquefaciens* subsp. *plantarum* (protease-producing bacterium), *S. maltophilia* (cellulase-producing bacterium) and *E. ludwigii* (alginate lyase-producing bacterium).

3.3.2. Viability in commercial pellets

Total viable cells (TVC) of *B. amyloliquefaciens* subsp. *plantarum* in the feed counted daily for 7 days showed significant differences, F=966.455, df 6,7, p <0. On day 1, the TVC of the bacterium was recorded at 6.8 Log CFU g^{-1} and after 24 h incubation at 4^{o}C

dropped sharply to 5.0 Log CFU g^{-1} (day 2). On day 3, the TVC continuously decreased from 5.0 log CFU g^{-1}feed to 4.8 log CFU g^{-1} feed. Thereafter, there was no significant difference between day 3 and day 4; between day 4 and day 5 as well as between day 6 and day 7. However, there was a slight decrease from day 3 to day 7, Figure 9. In general, the TVC decreased significantly in the first 24 h incubation, and appeared to be quite stable thereafter.

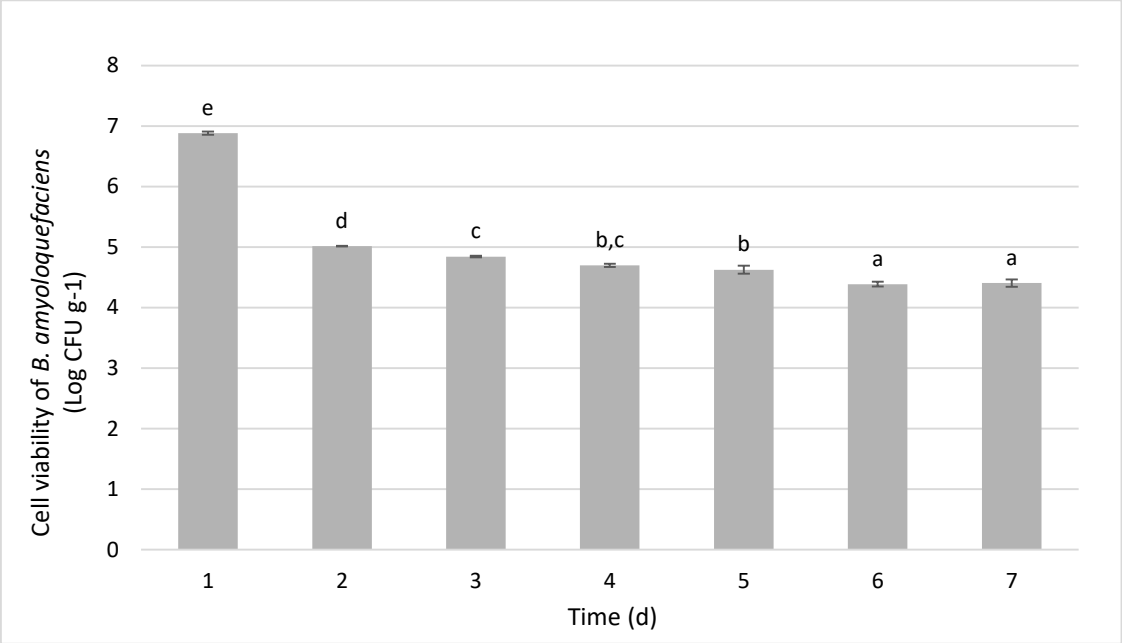


Figure 9. The changes in viable cells of *B. amyloliquefaciens* subsp. *plantarum* which was impregnated in commercial pellets and stored at 4 ºC for 7 days. Values are means with the standard deviation of two replicates. The different letters indicate that there were significant differences in cell viability, $P<0.05$.

TVC of *S. maltophilia* in the pellet counted on day 1, 2, 3, 4, 5, 6 and day 7 were significantly different, $F=14.120$, df 6,7, $P<0.05$. However, the difference was observed due to a slight decrease in the last day (day 7). Meanwhile, there was no significant different in TVC from day 1 to day 6. In general, the viability of *S. maltophilia* in the pellet was quite stable,

decreased only 0.2 log unit (from 7 log CFU g^{-1} to 6.8 log CFU g^{-1}) after being stored for 7 days at 4 ºC, Figure 10.

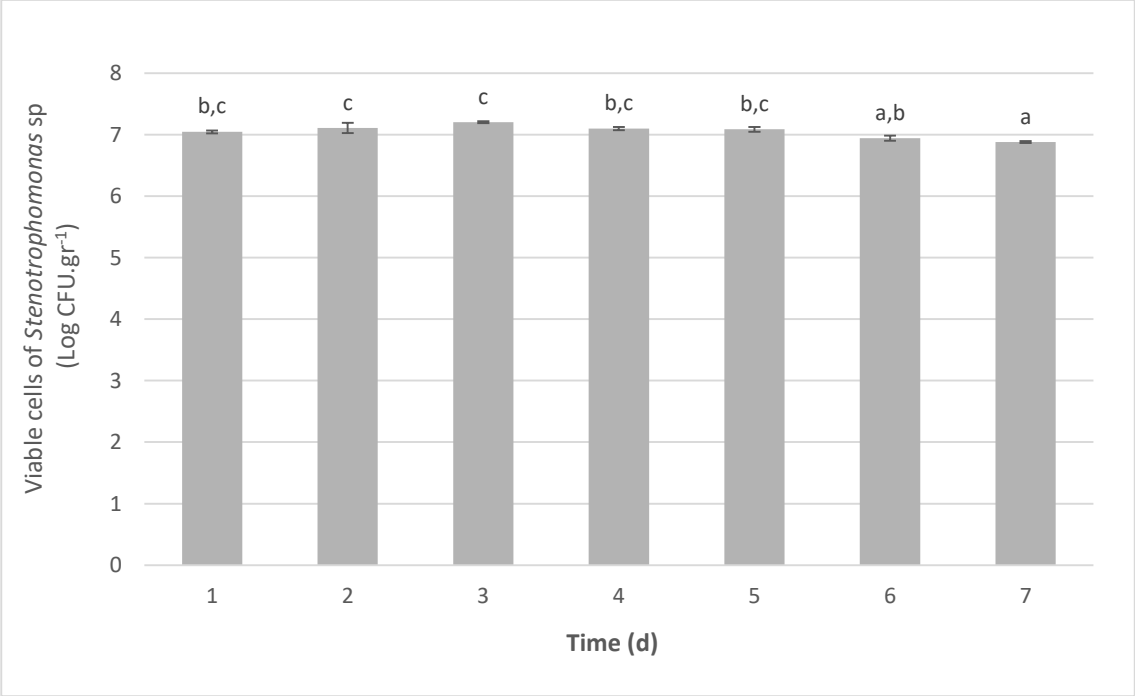


Figure 10. The change of viable cells of *S. maltophilia* impregnated in commercial pellet and stored for 7 days in 4 ºC. Values are means of viable cells (log CFU g^{-1}) with standard deviations of two replicates. The different letters indicated that there were significant differences in cell viability, $P<0.05$.

TVC of *E. ludwigii* in the commercial pellets counted on day 1, 2, 3, 4, 5, 6 and day 7 significantly different, $F=7.60$, df 6,7, $P=0.009$. TVC appeared to increase the first 24 incubation, from 7.1 to 7.3 log CFU g^{-1}. Then, the TVC dropped 0.3 log unit to 7 log CFU g^{-1} on day 3. Afterwards, the TVC counted on day 3, 4, 5, 6, and day 7 were not significantly different, Figure 11.

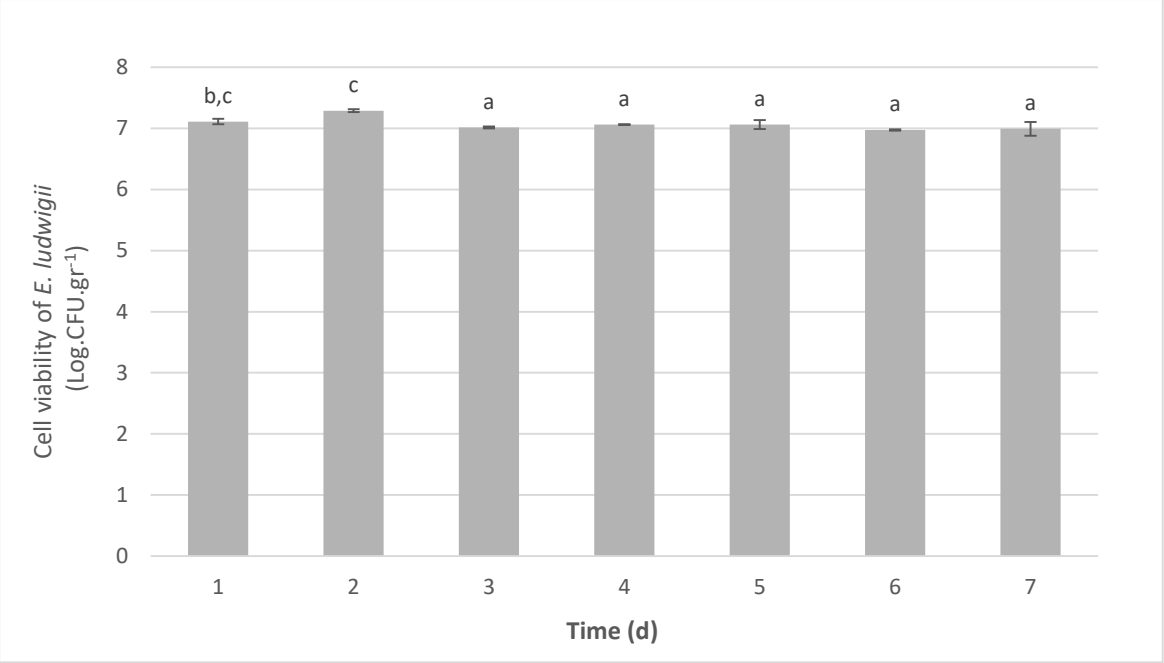


Figure 11. The change of viable cells of *E. ludwigii* impregnated in commercial pellets and stored in 4 ºC for 7 days. Values are means of total viable cells (log CFU g^{-1}) with standard deviations of two replicates. The different letters indicated that there were significant differences in cell viability.

Overall, two-thirds of the selected bacteria, *S. maltophilia* and *E. ludwigii*, had good viability on the manufactured abalone pellets, indicated by the stability of viable cells after 7-day incubation at storage temperature.

3.3.3. Viability in seawater

There were significant differences in the number of viable cells of *B. amyloliquefaciens* subsp. *plantarum* and *Enterobacter ludwigii* after exposure to seawater (32 ppt) at room temperature, Figure 12**.** The number of viable cells of *B. amyloliquefaciens* subsp. *plantarum* appeared to be 0.9 log unit lower after 6 h exposure compared to the initial viability (0 h). Conversely, the viable cells of *Enterobacter ludwigii* were increased 0.6 log unit after 6 h exposure, which suggests that this strain could grow in the rearing water. There was no significant difference in the number of viable cells of *S. maltophilia* between 0 h and

6 h exposure in the seawater, t=0., df 3, p = 0.97. Survival of *E. ludwigii* and *S. maltophilia* in seawater was very good, and reasonable for *B. amyloliquefaciens* subsp. *plantarum*.

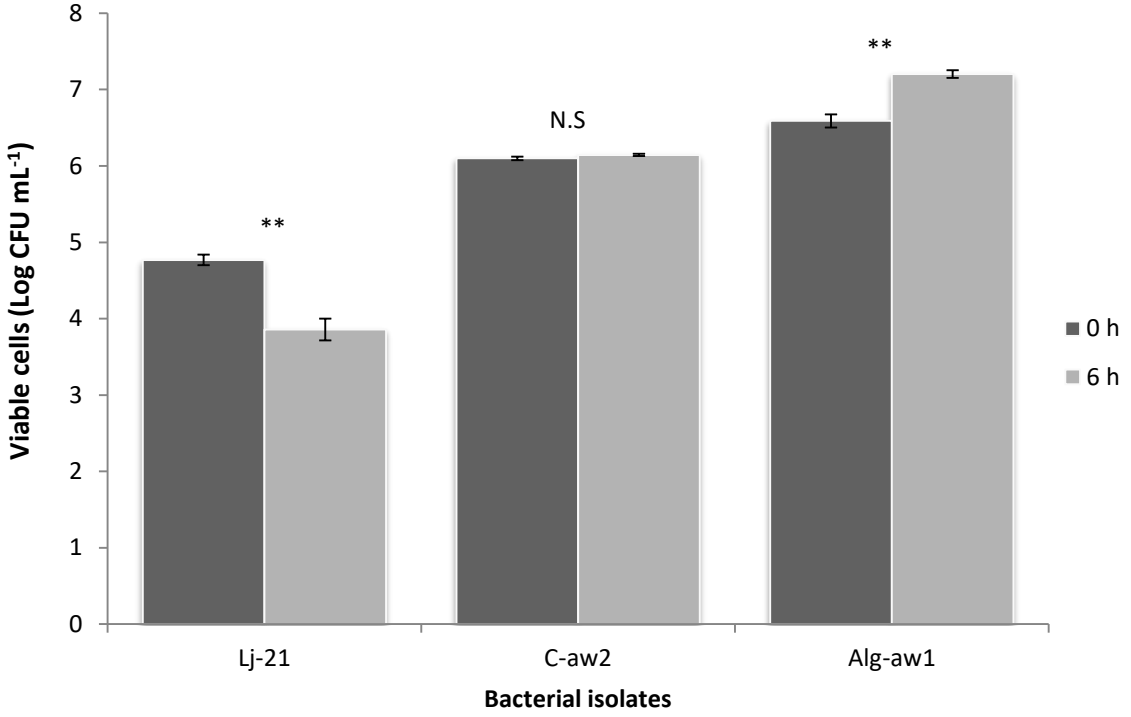


Figure 12. The changes in the number of viable cells of three enzyme-producing bacteria at 0 h and after 6 h exposure to seawater (32 ppt) at room temperature. The bars are means with standard deviations of 2 replicates. *Asterisks* indicate that there was a significant difference in cell viability at 0 h and 6 h after the exposure, ** = P<0.05. Lj-21: *B. amyloliquefaciens* subsp. *plantarum*, C-aw2: *S. maltophilia* and Alg-aw1: *E. ludwigii*.

3.3.4. Viability in SSJ

All tested species seemed to have a high tolerance to low pH in the SSJ, Figure 13. There were no significant differences in the number of viable cells of *B. amyloliquefaciens* subsp. *plantarum* and *S. maltophilia* at 0 h and after 3 h exposure in the simulated stomach juice, t= 0.19, df 3, P= 0.87. However, there was significantly higher numbers of viable cells after

3 h exposure in the simulated stomach juice for both *E. ludwigii*, P <0.05. The number of viable cells was increased from 6.8 Log to 7.3 log units for *E. ludwigii*.

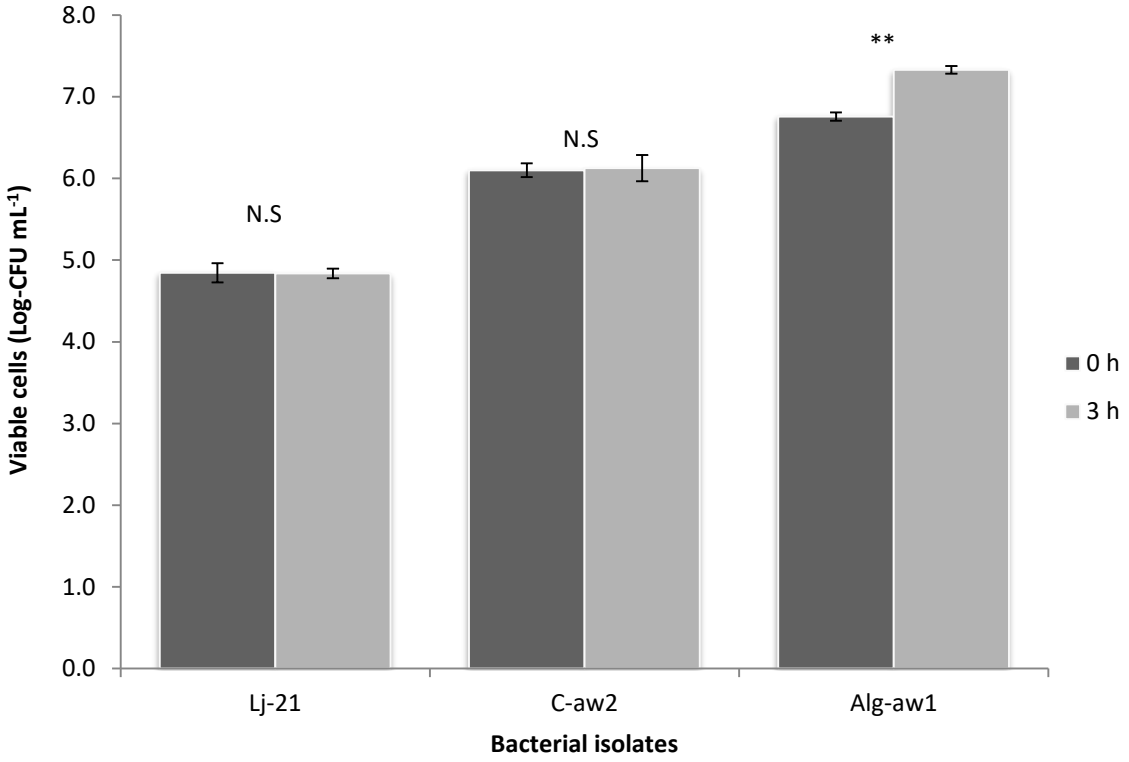


Figure 13. The change of viable cells in three enzyme-producing bacteria at 0 h and after 3 h exposure in the SSJ at room temperature. Bars indicate means with standard deviation of two (10 mL-bottle SSJ) replicates. *N. S* indicates no significant difference in cell numbers (CFU.mL^{-1}) between 0 h and after 3 h exposure. *Asterisk* indicates that there was a significant difference, **= P<0.05. Lj-21: *B. amyloliquefaciens* subsp. *plantarum*, C-aw2: *S. maltophilia*, and Alg-aw1: *E. ludwigii*.

Overall, the 3 bacterial strains showed good tolerance and viability in the SSJ and SIJ of abalone. In fact, *E. ludwigii* appeared to grow in the SSJ and in seawater.

3.3.5. Viability in SIJ

There was a significant decrease in the number of viable cells of *B. amyloliquefaciens* subsp. *plantarum* after 4 h exposure into the SIJ which suggests its sensitivity to the bile salt, t=31.5, df,1, P= 0.02. The number of viable cells decreased from 5.8 log at 0 h to only 2.5 after 4 h exposure, Figure 14. Meanwhile, the number of viable cells of *S. maltophilia* showed slight increase 0.3 log units after 4 h exposure in the simulated intestinal conditions (t=5.75, df 3, P=0.01). the other tested bacterium, *E. ludwigii* showed no significant difference in viable cells after 4 h incubation (t=0.234, df 3, P=0.830).

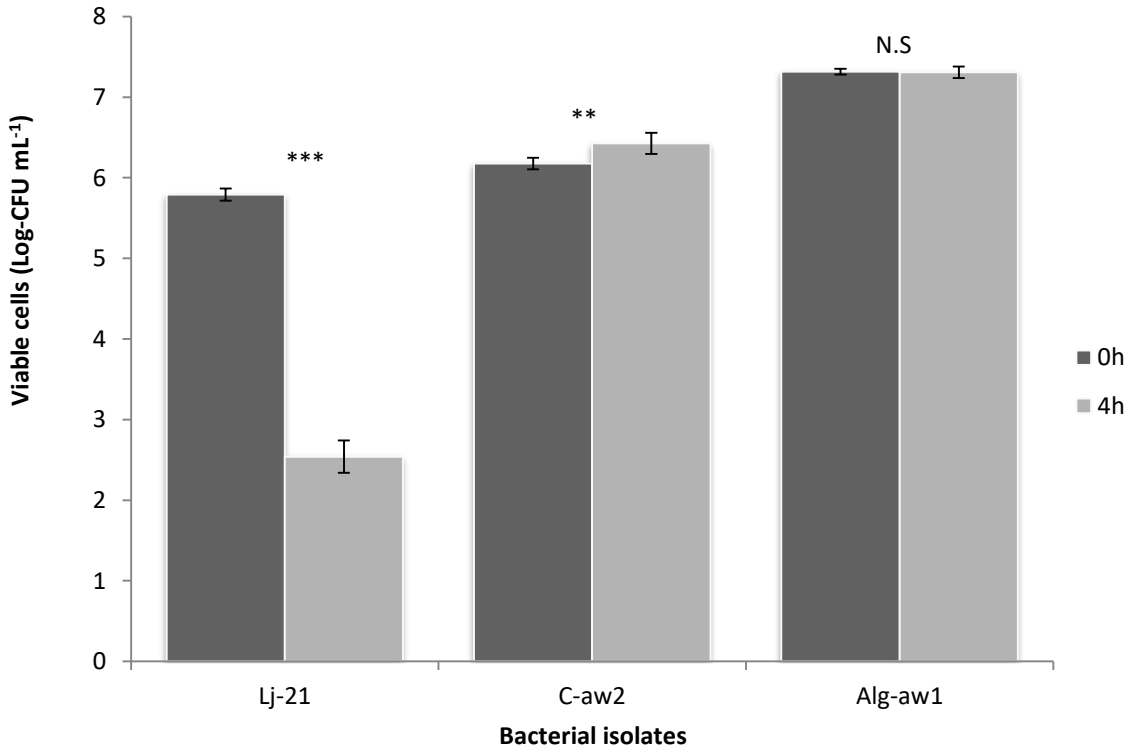


Figure 14. Changes in the viable cells of three enzyme-producing bacteria at 0 h and after 4 h exposure in the SIJ. Bars indicate means with standard deviations of two replicates. *N.S* is no significant difference in viable cells between 0 h and after 4 h exposure, (P>0.05). *Asterisks* indicate there was a significant difference, **=

P<0.05; and ***=P<0.01. Lj-21: *B. amyloliquefaciens* subsp. *plantarum*, C-aw2: *S. maltophilia,* and Alg-aw1: *E. ludwigii*.

3.3.6. Toxicity assay

There was no significant difference in survival rate of juvenile abalone among all treatment groups. After the abalone were fed at 1.5 %BW day^{-1} with either commercial pellets impregnated with ~1.0 x 10^9 CFU mL^{-1}, no mortality was observed in juvenile abalone fed with either one of these three bacteria (*B. amyloliquefaciens* subsp. *plantarum, S. maltophilia,* or *E. ludwigii*). In addition, there were no signs of disease observed in all juvenile abalone during the 14-day experiment.

3.3.7. Susceptibility to antibiotics

All tested strains were observed to be susceptible to chloramphenicol (10 and 30 µg mL^{-1}), ampicillin, and carbenicillin. *B. amyloliquefaciens* subsp. *plantarum* was shown to be susceptible to all tested antibiotics. *S. maltophilia* was resistant to vancomycin, and novobiocin. *E. ludwigii* was resistant towards vancomycin, oxytetracycline and novobiocin, Table 13.

Table 13. Susceptibility of three enzyme-producing bacteria to antibiotics.

Antibiotics	*B. amyloliquefaciens* subsp. *plantarum*	*S. maltophilia*	*E. ludwigii*
Chloramphenicol 10 µg/mL	-	-	-
Chloramphenicol 30 µg/mL	-	-	-
Ampicillin 10 µg/mL	-	-	-
Carbenicillin 100 µg/mL	-	-	-
Vancomycin 60 µg/mL	-	+	+
Oxytetracyclin 30 µg/mL	-	-	+
Novobiocin 5 µg/mL	-	+	+

3.4. Discussion

3.4.1. Digestibility and the role of digestive enzymes

Digestibility is defined as a proportion of diets which can be extracted and absorbed by cultured animals (Bedford, 1996; Kolkovski, 2001; Ray et al., 2012). Ray et al. (2012) reviewed that digestibility of diets depends mainly on three factors: ingested diets and their susceptibility to digestive enzymes; activity of digestive enzymes; length of the time to which the diets are exposed to digestive enzymes. These factors suggest that enzymes play central roles in feed digestibility. Based on their sources, digestive enzymes can be grouped into endogenous enzymes (produced by the animal hosts) and exogenous enzymes (produced by gastrointestinal indigenous microbes). Endogenous enzymes are released from oesophangeal and intestinal mucosal tissues, and organs such as pyloric caeca, and pancreas (De Silva and Anderson, 1994). The presence of endogenous digestive enzymes in fish has been reported in numerous cultured species including Grass carp (*Ctenopharyngodon idella*) (Das and Tripathi, 1991), African bony-tongue fish, *Heterotis niloticus* (Fagbenro et al., 2000), and abalone (Erasmus et al., 1997). However, the production of endogenous enzymes depends on may factors such as: cultured species, developmental stages, and ambient temperatures (Kitamikado and Tachino, 1960; Cahu and Infante, 2001; Kamaci et al., 2010; Miegel et al., 2010). Other studies suggest that young animals with undeveloped digestive systems, as well as several important cultured adult species, lack certain digestive enzymes in their intestinal tracts (Bondi and Spandorf, 1954; Fish, 1960; Stickney and Shumway, 1974; Arias et al., 2007; Li et al., 2008). As consequence, these animals are unable to digest nutrients in feed and this is frequently associated with the poor digestibility of diets. Furthermore, undigested feed ingredients such as soluble fibers can give adverse effects to cultured animals, such as increasing the viscosity in small intestine, impeding the digestion

of nutrients resulting in growth reduction and several digestive disorders (Bromley and Adkins, 1984; Francis et al., 2001).

The addition of digestive enzymes to feed have been considered as a worthy practice to enhance feed digestibility of cultured animals. There are five types of enzymes which are currently dominant in the animal feed markets: (i) fiber-degrading enzymes, (ii) protein-degrading enzymes, (iii) starch-degrading enzymes, (iv) lipid-degrading enzymes, and (v) phytate-degrading enzymes. Additional enzymes are required by abalone including caraginase, agarase and alginate lyase (Erasmus et al., 1997). Nonetheless, the addition of digestive enzymes is considered to be expensive and easily denaturate in water.

The common approach is the use of digestive enzyme-producing bacteria. There are several studies which have documented the ability of numerous bacterial strains associated with intestinal tracts of aquatic species to produce digestive enzymes (Ray et al., 2012; Hadi et al., 2014). In fact, in several aquatic species which are considered to produce digestive enzymes endogenously, it has been presented that the enzymatic activity was due to the help of indigenous bacteria (Shcherbina and Kazlawlene, 1971; Bairagi et al., 2002; Saha et al., 2006). For instance, freshwater fish, *Ictalurus punctatus*, exposed to antibiotics (streptomycin) for 24 h showed no cellulase activity, while control fish (unexposed to the antibiotic) had cellulase activity (Stickney and Shumway, 1974). This approach is increasingly popular nowadays because it has been regarded as ecofriendly technology. Therefore, many studies have been conducted to isolate and screen digestive-enzyme producing bacteria as probiotic candidates.

3.4.2. Potential capacity to colonize the intestinal tract

Probiotic candidates which are aimed at helping feed digestion are required to be viable in the GIT of their hosts (Verschuere et al., 2000a; Nayak, 2010b; Martinez Cruz et al., 2012). To be viable in the GIT, the probiotic candidates should be able to tolerate low

pH and bile salts (Nikoskelainen et al., 2001b; Fjellheim et al., 2010; Geraylou et al., 2014). Thus, three bacterial isolates (the isolate from each enzyme group with the highest enzyme activity) were further studied for their viability in the simulated stomach and intestinal conditions. The results showed that the *E. ludwigii* and *S. maltophilia* displayed good viability in the simulated stomach and intestinal conditions, indicated by no biologically significant difference in the number of viable cells at 0 h and after 3 or 4 h incubations in the low pH of simulated stomach and in bile salts of the simulated intestinal juice. These results could be initial indicators for capacity to colonize intestinal tract, acknowledging some bacterial sensitivity to low pH in the stomach and bile salt in the intestines (Giannella et al., 1972; Borriello et al., 1985). In fact, *E. ludwigii* appeared able to grow in simulated stomach juice, and *S. maltophilia* grew in the simulated intestinal juice, indicated by the significant increase in viable cells after either 3 h or 4 h incubation. Regarding the capacity to tolerate bile salt, these bacteria might produce bile salt hydrolase to break down bile salt (Du Toit et al., 1998) or produce a protective coating of exopolysaccharide (Roberts and Powell, 2005) to protect them from bile salt.

However, *B. amyloliquefaciens* subsp. *plantarum* showed significant decrease in cell viability after being incubated in commercial pellet, rearing water and SIJ. These results may indicate that the bacterial isolate was very sensitive to storage temperature (4^{o}C), salinity, and bile salt in the SIJ. However, *B. amyloliquefaciens* subsp. *plantarum* can be administered in the form of spores, as other genus *Bacillus* (Prieto et al., 2014; Reda and Selim, 2015). A study which investigate the formation of spore and recoverability of *B. amyloliquefaciens* subsp. *plantarum* spore in commercial pellet, and SIJ of abalone needs to be performed.

A toxicity assay indicated that the three probiotic candidates appeared to be harmless to juvenile abalone. However, previous studies indicated that *S. maltophilia* is an

opportunistic pathogen (Denton and Kerr, 1998; Looney et al., 2009). Since the toxicity study was performed in normal condition, the harmful effect of the bacterial species might not appear yet. Thus, further toxicity assay in which abalone are exposed in stress conditions should be performed and followed by a challenge test with *S. maltophilia*. This study would indicate whether the bacterial strain is an opportunistic pathogen or harmless to abalone.

3.4.3. Administration Method

Maintaining probiont viability in target sites is a major challenge in probiotic application (Wang et al., 2008); thus, the delivery method is a critical step to introducing bacteria. Common delivery methods of probionts into aquatic animals are by direct addition to rearing water (Verschuere et al., 2000a) or by mixing with diets (Carnevali et al., 2004). Several researchers considered that rearing water was a proper medium to introduce probionts to certain hosts (Ringø, 1999; Suzer et al., 2008; Avella et al., 2010). However, other authors criticized the efficacy of this method as it was applicable only in a closed aquaculture system such recirculation systems. Meanwhile, this technique is considered to be inappropriate when applied in open-aquaculture systems such as ponds, sea/lake cages and other flow-through rearing systems (Suzer et al., 2008; Merrifield et al., 2010b). The other common method is by mixing probionts into diets (Phianphak et al., 1999; Chiu et al., 2007; Iehata et al., 2009; Swain et al., 2009; Iehata et al., 2010; Kosin and Rakshit, 2010; Iehata et al., 2014; Maeda et al., 2014). This method can be applied in both open and closed aquaculture systems, even though some questions arise in terms of probiotic viability in the diets due to water content and nutrient availability in feed matrix, as well as being laborious in preparation.

Acknowledging these issues, these bacteria were assessed for their viability using both delivery methods, direct adding to rearing water and to a commercial pellet. The results showed that all tested bacteria had a good tolerance to the feed matrix, and in 32 ppt of

seawater. Two enzyme-producing bacteria, *S. maltophilia*, and *E. ludwigii* were even growing in the seawater, indicated by a significant increase in total cell viable numbers (log $CFU.mL^{-1}$) after 6 h incubation in the rearing water. In addition, these bacteria showed good viability in a commercial pellet matrix, characterized by no significant decrease in the number of viable cells after being stored in a cold room (4 ^{o}C) for 7 days. However, *B. amyloliquefaciens* subsp. *plantarum* showed a significant decrease in cell viability in both the feed matrix and the rearing water. These results may suggest that these enzyme-producing bacteria could be delivered via either the feed matrix or rearing water, though higher numbers of cells would be required for *B. amyloliquefaciens* subsp. *plantarum*.

3.4.4. The implication of enzyme-producing bacteria in aquaculture industries

The supplementation of enzyme-producing bacteria has been documented to improve feed digestibility, which results in better FCR and specific growth rates (SGR), as well as reducing the load of nutrient waste into the environment. The inclusion of enzyme-producing *Bacillus* spp. via feed decreased 0.2 unit of FCR and increased 0.4-unit protein efficiency ratio (PER), and 0.6 unit of SGR in rainbow trout fry (Bagheri et al., 2008) and 0.3 unit in common carp. In addition, other members of *Bacillus* (*B. subtilis, B. lincheniformis B. circulans)* have been reported to improve FCR and PER values in rainbow trout (Merrifield et al., 2010a), and in fingerlings of *Catla catla* (Bandyopadhyay and Das Mohapatra, 2009). The better values of FCR, PER and SGR not only could reduce nutrient pollution released to the environment, but also decrease the total amount of operational cost, as 60-80 % of the total production cost was coming from feed.

The incorporation of enzyme-producing bacteria may help to develop low-cost formulated diets. Shi et al. (2016), for instance, observed that no significant difference in the growth rate of carp was observed between carp receiving feed containing 6% fish meal and carp fed on a diet with 3 % fish meal supplemented with exogenous protease. This result

may suggest that the amount of fish meal, which is a major and increasingly expensive component of aquafeed, can be decreased. In addition, the use of fish meal can be partially replaced by plant protein sources such as soybean meal and microalgae, which are much cheaper than fish meal. The poor digestibility of plant-based diets, especially in monogastric fish due to the high cellulose content (Becker, 2007; Marshall et al., 2010), can be helped by the inclusion of the cellulase-producing bacteria (Mihranyan, 2011; De et al., 2015). These studies show that protease and cellulase - producing bacteria can be used as potential alternatives to lower the high cost of aquafeed. However, more research should be conducted to determine if the inclusion of these enzyme-producing bacteria reported in this present study could be used to either partially replace fish meal with plant based proteins or decrease the amount of fish meal, without reducing the growth of cultivated animals.

3.5. Conclusion

A total of 24 bacteria isolated from the GIT of fish and hybrid abalone displayed a capacity to produce digestive enzymes such as protease, cellulase or alginate lyase. Bacterial isolates which produce the highest enzyme activities were identified as *B. amyloliquefaciens* subsp. *plantarum* (protease), *S. maltophilia* (cellulase) and *E. ludwigii* (alginate lyase). Further characterizations indicated that these three bacterial strains were harmless to juvenile hybrid abalone and maintained high viability in the simulated stomach and intestinal juices. The results also indicated these bacteria had good viability in rearing water and commercial pellets, suggesting that these could be potential administering techniques to cultured animals. In addition, the present results may suggest that three bacterial strains could be potential probiotic candidates for aquaculture industries.

Chapter 4 : Screening and Characterization of Lactic Acid Bacteria as Probiotic Candidates

4.1. Introduction

The increasing productivity of abalone aquaculture has been followed by the emergence of various bacterial diseases such as *V. harveyi* (Jiang et al., 2013b), *V alginolyticus* (Cai et al., 2006), *V. parahaemolyticus* (Cai et al., 2007; Cheng et al., 2008), *Vibrio carchariae* (Nicolas et al., 2002) and *Vibrio* spp. (Kua et al., 2011). In general, antibiotics and vaccination have been frequently used for the treatment and prevention of these diseases. However, both approaches have been questioned due to safety and reliability issues. The use of antibiotics, for instance, potentially gives rise to strains of antibiotic-resistant pathogens (Miranda and Zemelman, 2002). Furthermore, residues may accumulate in animal flesh (Sahu et al., 2008), non-specific targeting may affect beneficial organisms and finally, antibiotics may cause suppression of immune systems (Sahu et al., 2008). Meanwhile, the use of vaccination has been questioned in terms of reliability, especially when applied to animals with less-developed immune systems, such as abalone and larval stages of vertebrates (Allen et al., 2014). Therefore, considerable effort has been expended to find alternative approaches which are more environmentally friendly and more beneficial to the animals. One of the most promising approaches is the use of beneficial bacteria; a process generally known as probiosis. The manipulation of the gut microbiota via dietary supplementation of probionts is an alternative strategy to treat and prevent bacterial diseases.

The awareness of consumers of healthy diets has stimulated the development of probiotic approaches in the last few decades. In aquaculture industries, studies on probionts mostly focus on Gram-positive bacteria, such as LAB. The administration of LAB was reported to exclude various bacterial pathogens through several mechanisms including production of antimicrobial compounds, outcompeting pathogens for nutrients or adhesion sites in gut mucosa (Nikoskelainen et al., 2001b), and enhancement of hosts' immune responses (Panigrahi et al., 2005; Chiu et al., 2007). In terms of antimicrobial compounds,

LAB were reported to excrete various antagonistic compounds such as bacteriocins (Verschuere et al., 2000a; Lin et al., 2013), bacteriolytic enzymes, hydrogen peroxide (Verschuere et al., 2000a) and organic acids (Goncalves et al., 1997; Vazquez et al., 2005). Among the excreted antimicrobial compounds, many researchers take considerable interest in bacteriocins due to their inherent characteristics, such as being generally recognized as safe (GRAS) substances, being inactive and nontoxic to eukaryote cells, pH and heat tolerant, and having a relatively broad antimicrobial spectrum against many pathogens (Hwanhlem et al., 2014).

There are many species of LAB have been documented to produce bacteriocins active against various aquaculture pathogens (Hwanhlem et al., 2014; Iehata et al., 2014; Maeda et al., 2014; Dash et al., 2016; Sha et al., 2016). However, *Pediococcus acidilactici* is the only authorised/registered probiotic for aquaculture in the European Union (EU) (https://en.engormix.com). Therefore, this species has been one of targeted bacterium for isolation and screening for bacteriocin production. Among bacteriocins produced by *P. acidilactici*, pediocin PA-1 gains a considerable interest for many researchers (Chikindas et al., 1993; Cintas et al., 1995; Devi and Halami, 2011). This might be due to pediocin PA-1 having a broad inhibitory spectrum (Cintas et al., 1995).

Besides antimicrobial production, there are several criteria that microbes need to fulfill to selected as probionts, including the capacity of probiotic candidates to tolerate and colonize the GITs of the animal hosts. The potential capacity to colonize the GITs could be evaluated by their ability to tolerate the stomach and intestinal environment (Geraylou et al., 2014), and their adhesion capacity as well as the ability to grow in the intestinal mucus (Vine et al., 2004). Therefore, this study was aimed at the screening of indigenous LAB isolated from the GITs of aquatic animals for antimicrobial activity against eight bacterial pathogens,

and to investigate their capability to inhabit and colonize the GITs of aquatic animals by means of *in vitro* studies.

4.2. Material and Method

4.2.1. Bacterial strains and media

This study used 206 endogenous LAB previously isolated from the GITs of teleosts and molluscs (result of chapter 2). The LAB strains were firstly revived from glycerol stocks by subculturing in 5 mL MRS broth. The inoculated broth was incubated anaerobically at room temperature for 24 h. Afterward, purity of LAB was checked by plating on MRS agar according to the protocol in chapter 2. A well-separated colony was picked with a sterile loop and replated on MRS agar plate for further use.

4.2.2. Screening for productions of antimicrobial compounds

The capacity of these LAB to produce antimicrobial compounds against bacterial pathogens was investigated using either a microtiter plate assay or an agar well-diffusion assay. In general, both screening assays used extracellular product of each LAB.

4.2.2.1. Preparation of cell-free supernatant (CFS) and neutralized cell-free supernatant (CFSn)

A single colony of pure LAB isolate was picked and inoculated into 5 mL MRS broth. The inoculated broth was then placed inside an anaerobic jar with an anaerobic sachet (AN0035, Oxoid), and incubated for 24 h at room temperature. Afterwards, the bacterial cells were harvested by centrifugation at 13,000 x *g* for 1 min and washed with PBS (pH 7.2). Cell concentration was adjusted at ~1.0 x 10^8 CFU mL^{-1} by setting OD_{600} nm at 0.15-0.2. Thereafter, 100 µL of the aliquot was inoculated into 10 mL of MRS broth and incubated anaerobically at room temperature (22 ± 1.0 ^oC) for 24 h. The broth culture was centrifuged

at 13,000 x *g* for 10 min. at 4 ºC, and supernatant was collected in a sterile tube. After measuring its pH, one-half of the supernatant was approximately neutralized by adjusting its pH to 6.5-6.8 by adding 1 M sodium hydroxide (NaOH) in order to diminish the antimicrobial influence of organic acids. Meanwhile, the other half of the supernatant was left unadjusted. Thereafter, both supernatants were sterilized by filtering through a 0.22 µm Millex Syringe Filter (Millipore). The filtered neutralized supernatant (CFSn) and unneutralized supernatant (CFS) were stored at 4 ºC until further use.

4.2.2.2. Preparation of indicator pathogens

This study used eight bacterial pathogens: seven aquatic pathogens (*V. alginolyticus, V. parahaemolyticus, V. harveyi, V. anguillarum, V. proteolyticus, Yersinia ruckeri* strain UTYR 001A, and *A. hydrophila*) and one foodborne pathogen, *L. monocytogenes*. The seven aquatic pathogens were isolated from fish and become part of culture collections in the Aquatic Microbiology Laboratory of the IMAS Launceston. Meanwhile the food borne pathogen originated from food and part of collection in the Microbiology Laboratory of Human Life Science, University of Tasmania, Launceston. Each bacterial pathogen was subcultured in 5 mL of MH broth and incubated at either room temperature (22 ºC) or 37 ºC (only for *L. monocytogenes*) aerobically for 24 h. Thereafter, bacterial cells were harvested by centrifugation at 13,000 x *g* for 1 min. Bacterial cells were diluted in NSS and cell concentration was adjusted to approximately 1.0 x 10^6 CFU mL^{-1} for further use.

4.2.2.3. Microtiter-plate assay

This assay was performed according to a protocol developed by Ringo (2008), with slight modification. Briefly, 150 µL of sterilized MH broth was added into each well of a sterile 96-well microtiter plate (P7241, Sigma-Aldrich). The wells were then inoculated with a 10 µL suspension of each indicator pathogens (~10^6 CFU mL^{-1}). Afterwards, 50 µL of either CFS or CFSn was added into the wells and into 50 µL of sterilized MRS broth as the control.

Each treatment and control had four well replicates. Thereafter, the plate was incubated at room temperature aerobically, and the growth of each pathogen was monitored by measuring optical density (OD) at 650 nm wavelength using an Infinite M200Pro spectrophotometer (TECAN) every 3 h for 24 h. A total of 25 LAB were screened using the microtiter plate assay.

4.2.2.4.Agar-well diffusion assay

This assay was conducted according to a modified protocol of Tagg and McGiven (1971). Briefly, one mL of each pathogen aliquot (~ 1.0 x 10^6 CFU mL^{-1}) was spread onto MH agar, and air dried for 5 min. Subsequently, 6 mm diameter wells were aseptically bored in the agar using the base of a sterilized pipette, followed by the addition of 80 µL of either CFS or CFSn of each LAB strain, or sterilized MRS as the control in duplicate wells. Each treatment and control had duplicate wells. Then, the agar plate was incubated aerobically at room temperature and, after 24 h, the CFSn or CFS exhibiting clearance zone was measured. A total of 181 LAB were screened using the well-diffusion agar method. Two LAB strains exhibiting the broadest antimicrobial spectra, especially to 5 vibrios infecting abalone, were selected for further studies.

4.2.3. Detection of bacteriocin-encoding genes

DNA of the antagonistic LAB was extracted using the PowerSoil DNA isolation Kit (MoBio, USA). The presence of pediocin PA-1 – encoding gene was detected using one µL of the DNA and ae set of primers. The sequences of primers were Forward: PEDRPO (F): 5′-CAA GAT CGT TAA CCA GTT T-3′; PEDC1041 (R): 5′-CCG TTG TTC CCA TAG TCT AA-3′ (Todorov and Dicks, 2009). The amplification reaction was prepared and run at the following conditions: an initial denaturation, 94 ºC for 1 min followed by 35 cycles of 1 min at 94 ºC, 30 s at 50 ºC for 30 s, and 1 min at 72 ºC, and final extension at 72 ºC for 5 min.

4.2.4. Tolerance to SSJ

The capacity of LAB to colonize the GIT was evaluated according to a modified protocol of Geraylou et al (2014). In brief, fresh culture of each LAB strain was subcultured in MRS broth and incubated anaerobically for 24 h at room temperature. Then, the broth culture was centrifuged at 13,000 x *g* for 1 min. Bacterial cells were washed and resuspended in sterile PBS to a concentration of ~1.0 x 10^7 CFU mL^{-1}. Fifty µL of the suspension was inoculated in duplicates of 5 mL of a simulated stomach juice which contained 3 mg mL of pepsin and 0.85 % of normal saline solution (NSS), with pH 3.5. Afterwards, the mixture was incubated aerobically at room temperature for 24 h. Cell viability of each LAB strain was determined by plating onto MRS agar at 0 h (initial cell numbers) and after 3 h exposure.

4.2.5. Tolerance to SIJ

The viability of LAB strains in fish intestinal tracts was evaluated *in vitro* using a modified protocol developed by Geraylou et al., (2014). A fresh culture of LAB was diluted into sterile PBS and the cell concentration was adjusted to be approximately 1.0 x 10^7 $CFU.mL^{-1}$. Then, 50 µL of the suspension was inoculated into duplicates of 5 mL simulated intestinal juice with the following composition: 125 mM of NaCl, 7 mM of KCl, 45 mM of $NaHCO_3$, 0.3% bile salt, 3 g/L trypsin, and pH 7.4. Each LAB had two replicates. Afterwards, the culture was incubated at 21.3 ºC±2.4 ºC on a rotary shaker at 4 x *g* to simulate peristalsis. Samples were serially diluted and plated on MRS agar at 0 h and 4 h for cell viability.

4.2.6. Adhesion capacity to intestinal mucus

The mucus of the GIT was prepared according to Nikoskelainen et al. (2001b). In brief, the fresh GIT of an Atlantic salmon (chosen as a commonly available animal) was opened and rinsed with 10 mM PBS buffer (pH 7.2). Then, the inner surface of the GIT was scraped with a spatula and placed inside a tube. The scraped mucus was then weighed and

centrifuged at 10,000 x g for 30 min at 4 ^{o}C. The supernatant was collected and diluted in the PBS buffer (1:9). Afterwards, a 96-well microplate was coated with 150 µL of salmon intestinal mucus and left overnight at 4 ^{o}C. The wells were subsequently washed with the PBS to remove unbound mucus. Hundred µL of fresh LAB isolate (~ 1.0 x 10^{8} CFU mL^{-1}) was added to each well, with 6 replicates. After 1 h, non-adherent bacteria were removed; meanwhile, the adherent bacteria were fixed for 20 min at 60 ^{o}C and stained with crystal violet for 45 min. The wells were washed with PBS to remove excess stain. Thereafter, the stain was released from the attached bacterial cells by washing them with 100 µL of 20 mM acetate buffer, pH 4.3. Total bacterial adherence was measured in terms of optical density (OD) at 600 nm wavelength and the percentage of bacterial cells which attached to the intestinal mucus was calculated with the formula (Geraylou et al., 2014):

$$AC\ (\%) = \frac{(A_1 - A_{Ctrl})}{A_0} \times 100$$

where:

AC : Adhesion capacity (%)

A_{Ctrl}: The absorbance values of the control (stained intestinal mucus)

A_0 : The absorbance values of the tested LAB used in the assay (~10^{8} CFU mL^{-1})

A_1 : The absorbance values of the LAB attached to the intestinal mucus.

4.2.7. Growth in intestinal mucus

Ten µL of overnight culture LAB was inoculated in 2.5 mL of diluted mucus in eight replicates (~1.0 x 10^{6} CFU mL^{-1}) and incubated aerobically at 22 ^{o}C for 48 h. Wells with only diluted mucus were also used as a control. The growth of tested bacterium was measured in terms of optical density (OD_{600}) in 4-h intervals for 48 h using an Infinite M200 Pro spectrophotometer. Then, growth profiles including specific growth rate (μ) were determined according to Vine et al. (2004), with some adjustments, Figure 15.

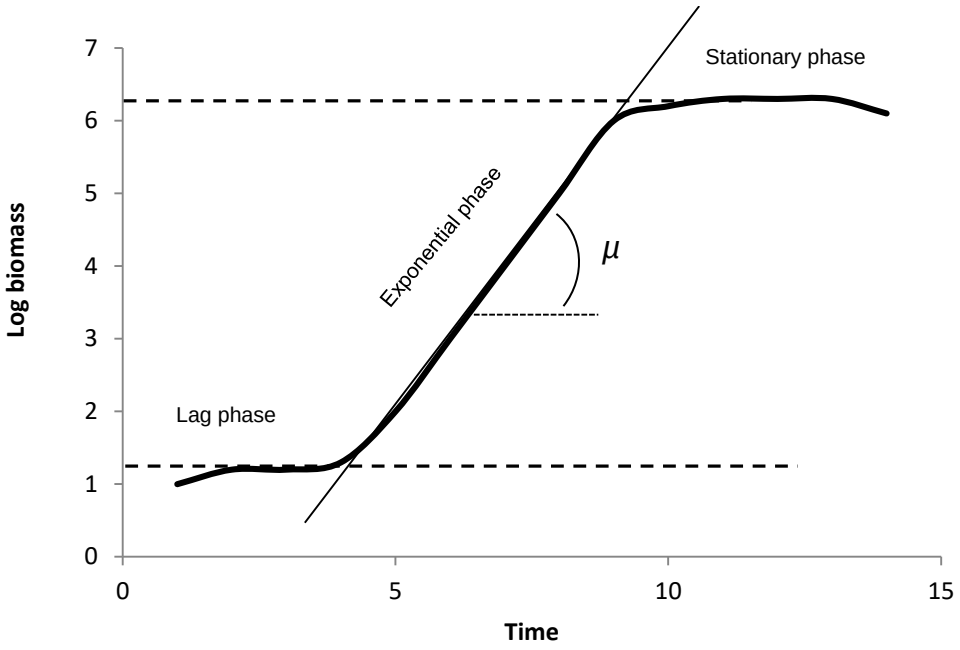


Figure 15. Growth curve of bacteria and its phases. The specific growth rate (μ) of each LAB strain was calculated at the exponential phase.

4.2.8. Antibiotic Resistance

The susceptibility of two LAB strains (with broad spectrum antibacterial activity against pathogenic vibrios) to chloramphenicol (8 µg/mL, 16 µg/mL, 32 µg/mL), which was one of the most common antibiotics previously used in aquaculture industries, was investigated. In brief, an MH agar plate was layered with one of the LAB strains. Then, an antibiotic disk was placed on the plate, followed by incubation at room temperature for 24 h. Thereafter, the diameters of the growth inhibition halos were measured.

4.2.9. Data analysis

The growth of pathogens treated with either CFS, CFSn or MRS broth in the microtiter plate assay was analyzed with a regression model to find the growth rate (slope). Afterwards, the growths were compared using one-way analysis of variance (ANOVA) to determine the significant difference ($P<0.05$) and continued with Post-Hoc Tukey test. Normality and homogeneity tests are performed before running the ANOVA test. Meanwhile, data of adhesion capacity and growth of the two pediococci in intestinal mucus

were compared using an independent sample t test at P<0.05. Data of cell viability in the simulated stomach and intestinal conditions were analyzed using Repeated Measures Define Factor with SPSS statistics software version 22.

4.3. Results

4.3.1. Antimicrobial compound – producing bacteria

A total of 22 LAB strains showed antimicrobial activity against one or more indicator pathogens: two active LAB strains were detected using a microtiter plate assay, indicated by the lower growth of indicator pathogens after being treated with the CFSn of LAB (Figure 16, & 17). Meanwhile, 20 LAB strains with antimicrobial activity were detected in the agar well-diffusion assay, indicated by a diameter of the clearance zone >10 mm (Figure 18 & Table 14).

Two LAB strains (*Lb. farraginis*, and *C. divergens*) exhibited inhibitory activity against three indicator pathogens: *V. anguillarum*, *V. harveyi*, and *A. hydrophila*. CFSn of *Lb. farraginis* significantly reduced the growth of *V. anguillarum* (F=3974, df, 2,9, P<0.01) and *V. harveyi* (F = 1703, df 2,9, P<0.01). The strongest inhibitory activity was observed in unneutralized CFS (pH, 4.8), in which no growth was observed in both indicator pathogens after 24 h incubation at room temperature. When the pH of the CFS was increased to 6.5 (CFSn), it showed reduction in inhibitory activity. However, the growth of indicator pathogens treated with the CFSn was still significantly lower than the growth of both indicator pathogens in the control. The growth of the two indicator pathogens treated with CFSn of *Lb. farraginis* was a half that of the control**,** Figure 16 a & b.

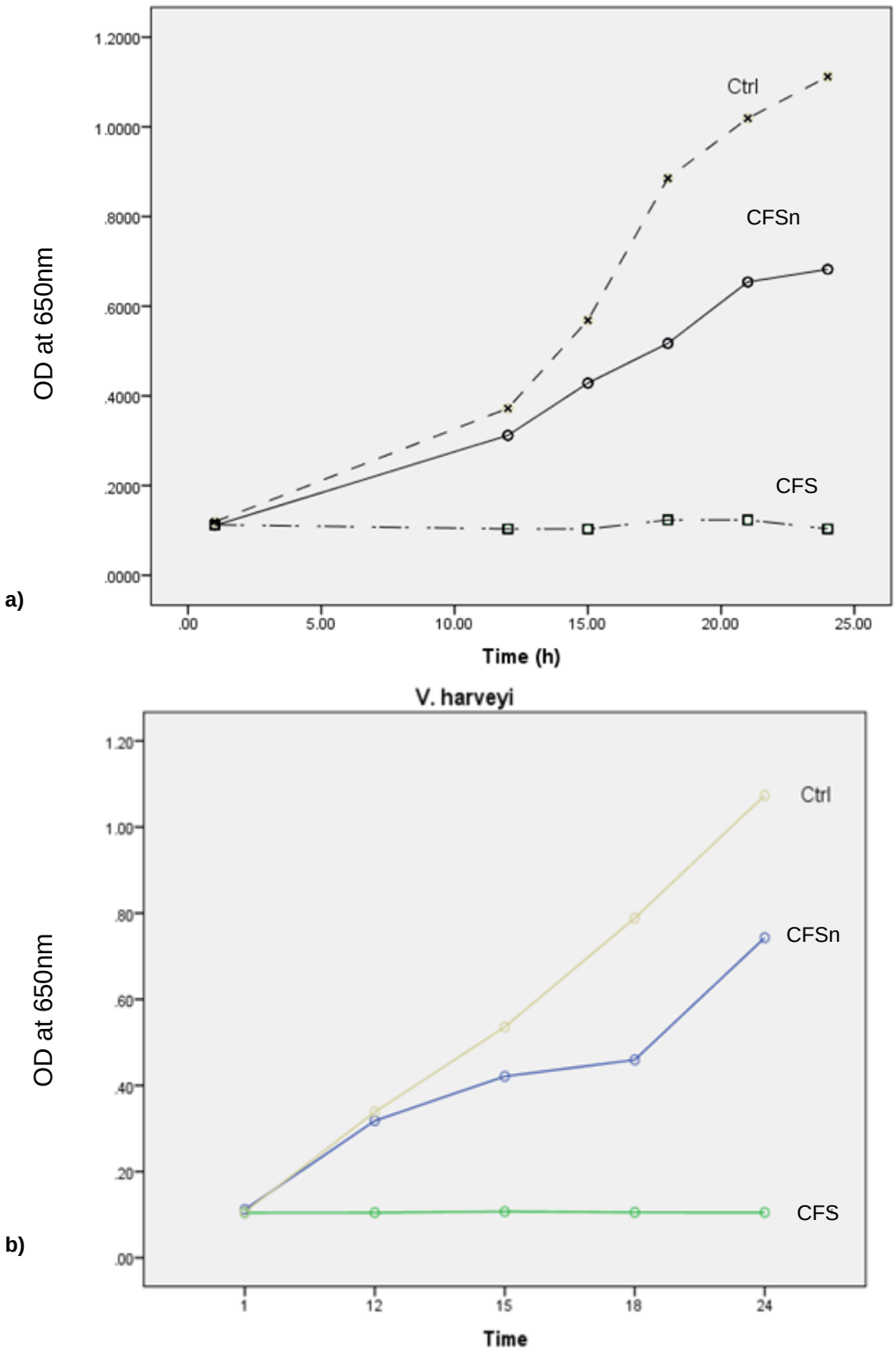


Figure 16. a). The mean optical density of *V. anguillarum* at 650 nm in mediums treated with extracellular metabolite product of *Lb. farraginis*). b) The growth of *V. harveyi* treated with either CFS, neutralized CFSn or sterilized MRS broth as the control (Ctrl). All values are means of pathogen growth, with standard deviation of four replicates. All values were calculated based on OD value at 650nm wavelength. Different letters indicate there was significant difference, $P<0.01$.

In addition, *C. divergens* isolated from the GIT of wild seabream showed inhibitory activity against *A. hydrophila*, Figure 17. The growth of *A. hydrophila* after being treated with either CFS or CFSn of *C. divergens* was significantly lower compared to growth of the pathogen in the control (F=500, df, 2,9, P=0.001). Overall, the strongest inhibitory activity was observed from CFS, and followed by the CFSn. CFS appeared to be bactericidal to *A. hydrophila* as no growth was observed; meanwhile, CFSn of *C. divergens* was bacteriostatic in which the growth of the pathogen was reduced from 0.044±0.003 (control) into 0.029±0.001 after being treated with the CFSn.

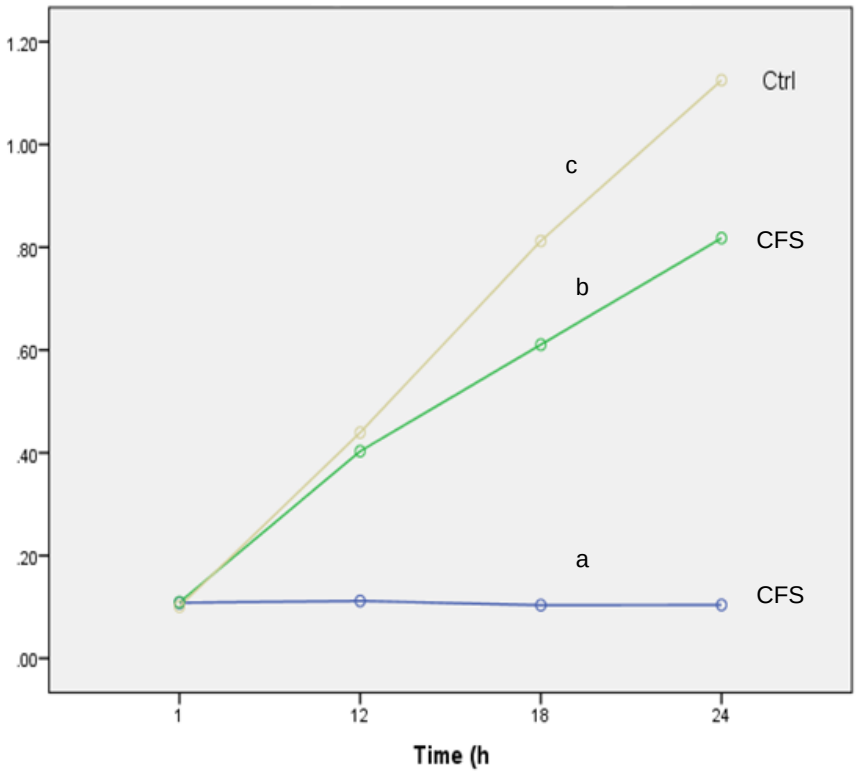


Figure 17. Growth of *A. hydrophila* in medium treated with either CFS, CFSn or MRS as the control. Growth was estimated based on the OD value at 650 nm. Superscripts with different letters indicate significantly different from one another, P<0.05.

Meanwhile, twenty LAB exhibited inhibitory activity against at least one bacterial pathogens using the well diffusion assay (Table 14), indicated by the diameter of the clearance zones being >10mm. The antagonistic LAB belonged to *L. farraginis*, *C.*

divergens, *C. gallinarum*, *P. acidilactici*, *P. pentosaceus*, *E. faecium* (2), *E. gilvus E. lactis* (2), *E. faecalis*, *E. thailandicus*, and *Leuc. mesenteroides*, *Lact. lactis* MA068, MA116, MA126, MA130, MA139, MA146, MA149.

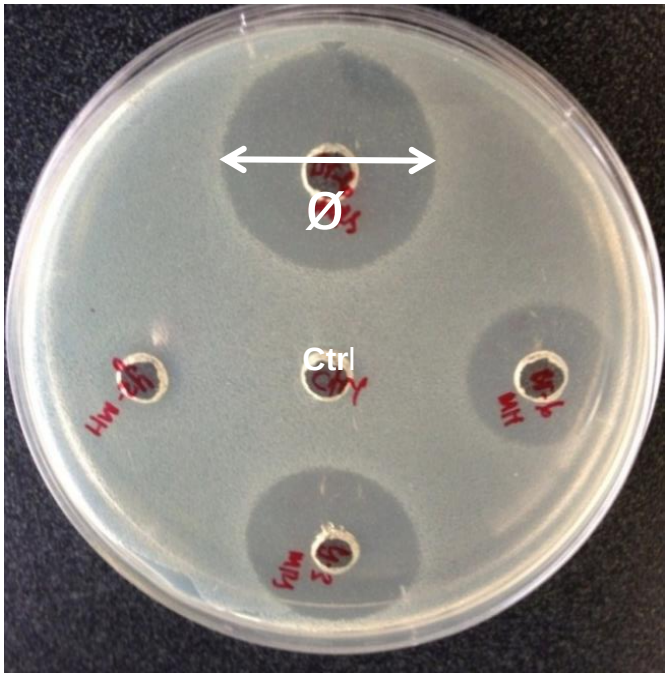


Figure 18. Inhibition zones of CFSn extracted from antimicrobial compound – producing bacteria on a Muller-Hinton agar plate seeded with *L. monocytogenes*. Ø is diameter of inhibition zone, and Ctrl is supernatant of sterilized MRS broth as the control.

Table 14. LAB isolates producing antimicrobial activity against aquatic and/or foodborne pathogens using either a microtiter plate assay or an agar-well diffusion assay.

No	LAB	Indicator Pathogens							
		AH	**VAng**	**VH**	**VAlg**	**VProt**	**Vpar**	**YR**	**LM**
1	*C. gallianrum* MA204	-	-	++	-	-	-	-	-
2	*C. divergens* MA108	m.p.a	-	-	-	-	-	-	-
3	*E. faecalis* MA187	-	-	+	-	-	-	+	-
4	*E. faecium* MA002	-	-	-	-	-	-	-	++
5	E. *faecium* MA115	-	-	-	-	-	-	-	++
6	*E. lactis* MA084	-	-	-	-	-	-	-	+
7	*E. lactis* MA056	-	-	-	-	-	-	-	+
8	*E. gilvus* MA122	-	-	-	-	-	-	+	+
9	*E. thailandicus* MA109	-	-	-	-	-	-	-	+
10	*Lb. farraginis* MA150	-	-	m.p.a	m.p.a	-	-	-	-
11	*Leuc. mesenteroides* MA064	-	-	-	+	-	-	-	-
12	MA068	-	-	-	+	-	-	-	++
13	MA116	+	-	-	-	-	-	-	+++
14	MA126	+	-	-	+	-	+	+	+
15	MA130	-	-	+	-	-	-	-	-
16	MA139	+	-	++	+	-	-	-	-
17	MA140	++	-	-	-	+	-	-	-
18	MA146	+	-	+	-	-	-	-	+
19	MA149	-	-	-	+++	-	-	-	-
20	MA176	+	-	+	-	-	-	+	++
21	*P. acidilactici* MA160	-	-	++	+	+	+	+	+
22	*P. pentosaceus* MA169	+	-	++	+	+	+	+	+

AH: *Aeromonas hydrophila,* **VAng***: Vibrio anguillarum,* **VH***: V. harveyi,* **VAlg***: V. alginolyticus,* **Vpro***: V. proteolyticus,* **Vpar***: V. parahaemolyticus,* **YR***: Yersinia ruckeri,* and **LM***: Listeria monocytogenes*. m.p.a: antimicrobial activity was detected using a microtiter plate assay. Values are means with standard deviation of two replicates for each LAB strain (+: 10-14 mm, ++: 15-19 mm and +++: >19mm diameter of inhibition zone).

4.3.2. pH of cell-free supernatant

The pH values of cell free supernatant extracted from LAB-inoculated MRS broth decreased, from 6.2 (pH of normal MRS broth) to 4-5 after 24 h incubation at room temperature, Table 15. Then, the pH of the CFS was adjusted to 6.5-6.8 (CFSn) in order to nullify the antagonistic effects of organic acids for further antimicrobial assay.

Table 15**.** Values of pH of CFS and CFSn used in screening of antimicrobial activity

No	LAB Identity	pH	
		CFS	CFSn
1	*C. divergens* MA108	5.5 ± 0.2	6.6 ± 0.2
2	*C. gallinarum* MA203	4.5 ± 0.1	6.6 ± 0.0
3	*E. faecalis* MA187	4.6 ± 0.3	6.6 ± 0.1
4	*E. thailandicus* MA109	4.9 ± 0.1	6.7 ±0.2
5	*E. lactis* MA084	4.5 ± 0.0	6.8 ± 0.0
6	*E. lactis* MA056	4.6 ± 0.0	6.7 ± 0.1
7	*E. faecium* MA002	4.5 ± 0.2	6.7 ± 0.2
8	*E. gilvus* MA122	4.9 ± 0.1	6.6 ± 0.1
9	*Leuc. mesenteroides* MA064	4.5 ± 0.2	6.6 ± 0.1
10	*P. acidilactici* MA160	4.3 ± 0.1	6.7 ± 0.2
11	*P. pentosaceus* MA169	4.3 ± 0.2	6.6 ±0.2
12	*Lb. farraginis* MA150	4.8 ± 0.4	6.8 ± 0.1
13	MA115	5.0 ± 0.2	6.5 ±0.0
14	MA116	4.8 ± 0.4	6.7 ±0.3
15	MA139	4.9 ± 0.4	6.7 ± 0.2
16	MA068	4.7 ± 0.1	6.7 ± 0.0
17	MA176	4.5 ± 0.1	6.7 ± 0.2
18	MA140	5.0 ± 0.1	6.7 ± 0.3
19	MA146	4.9 ± 0.0	6.6 ± 0.1
20	MA126	4.7 ± 0.2	6.8 ± 0.1
21	MA130	4.9 ± 0.3	6.6 ± 0.2
22	MA149	4.7 ± 0.2	6.7 ± 0.1

CFS: Cell-free supernatant, CFSn: neutralized cell-free supernatant

4.3.3. Amplification of bacteriocin-encoding genes

Two LAB strains (*P. acidilactici* MA160 and *P. pentosaceus* MA169) were selected for further studies due to their wide spectra of inhibitory activity against 5 vibrios (*V. anguillarum*, *V. harveyi*, *V. alginolyticus*, *V. proteolyticus*, and *V. parahaemolyticus*), four

of which have been reported to infect abalone. The possibility of both *Pediococcus* spp. carrying one of the bacteriocins produced by the pediococci was investigated by amplifying the pediocin PA-1 encoding gene. The result showed that the band size of the Pediocin PA-1-encoding gene from *P. acidilactici* MA160 and *P. pentosaceus* MA169 was ~ 500 bp, and 1,000 bp respectively, Figure 19.

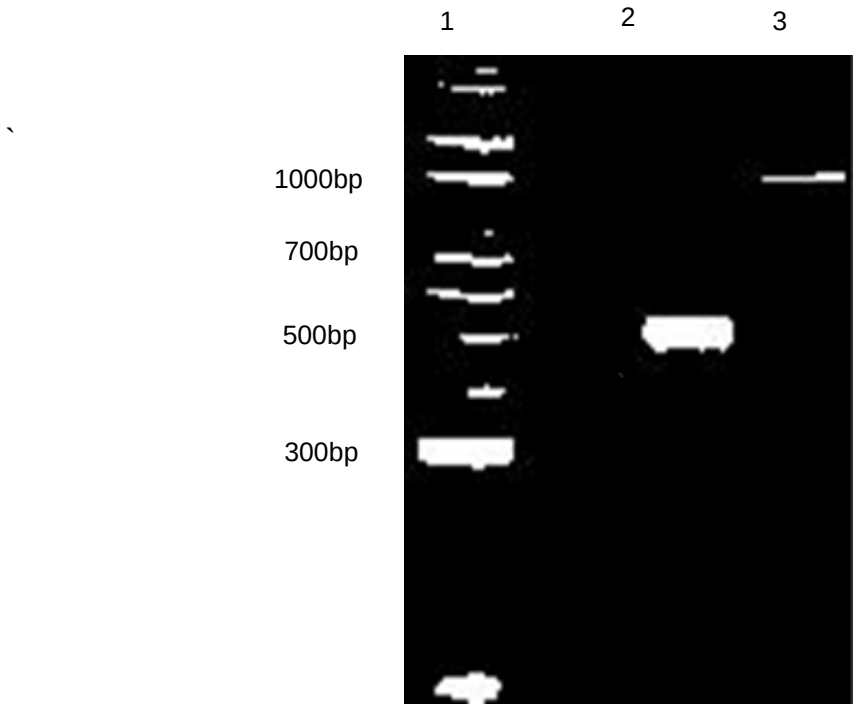


Figure 19. PCR products amplified using Bacteriocins-targeted primers. Lane 1 is the DNA marker (Bioline Hyper Ladder II). Lane two is the Pediocin-encoding gene detected from *P. acidilactici* MA160 with Lane 3 from *P. pentosaceus* MA169.

4.3.4. Tolerance to SSJ

The results showed that the two pediococci had high survival rates after 3 h exposure in the SSJ, shown in Figure 20. For *P. acidilactici* MA160, the number of viable cells was slightly decreased from 6.4 log unit at 0 h to 6.1 log unit after 3 h exposure, or only 0.3 log unit decrease. Meanwhile, the number of viable cell counted after 3 h exposure in the SSJ was no significant difference for *P. pentosaceus* MA169, ($P>0.05$).

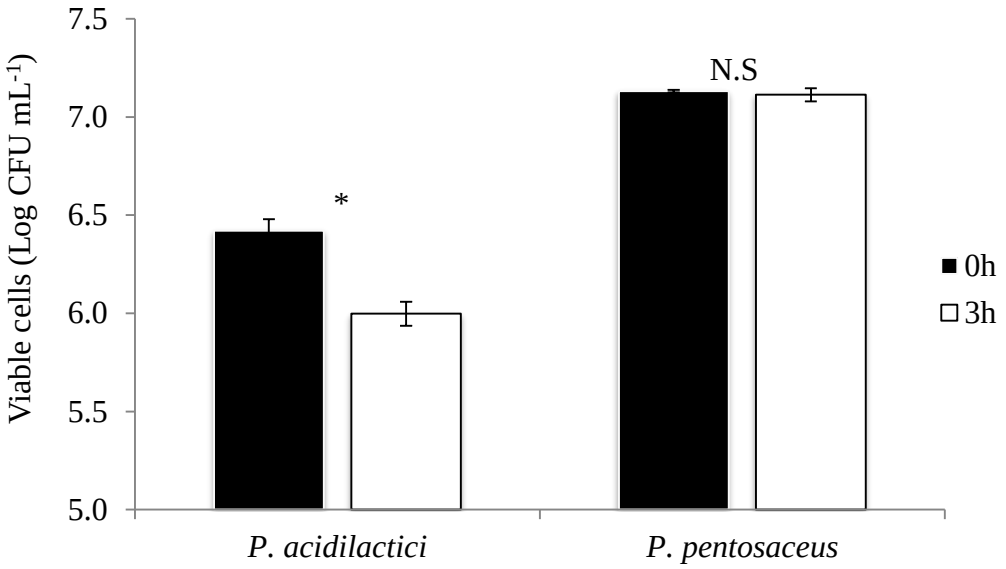


Figure 20. Cell viability of *P. acidilactici* MA160 and *P. pentosaceus* MA169 at 0 h and 3 h exposures in simulated stomach juice. All values are means with standard deviations of two replicates. *N.S* indicates no significant difference in cell numbers (log CFU mL^{-1}) between 0 h and 3 h exposure, and *asterisk* indicates there was significant difference in cell viability between 0 h and 3 h exposure, $P<0.05$.

4.3.5. Tolerance to SIJ

Both pediococci showed high viability after 4 h exposure in the SIJ, Figure 21. The cell counts of *P. acidilactici* MA160 were not significantly different at 0 h, and after 2 h and 4 h exposure in the SIJ ($P> 0.05$), although there was a slight decrease in viable cells after 4 h exposure from log7.01 (0 h) to log6.98 (2 h) and log6.97 (4 h). Meanwhile, the cell counts of *P. pentosaceus* MA169 were significantly decreased after 2 h and 4 h of exposure in the SIJ ($P<0.05$). It was observed that viable cells decreased 0.1 log units consecutively every 2 h, from 6.95 log units at 0 h to 6.86 log units after 2 h and 6.74 log units after 4 h exposure.

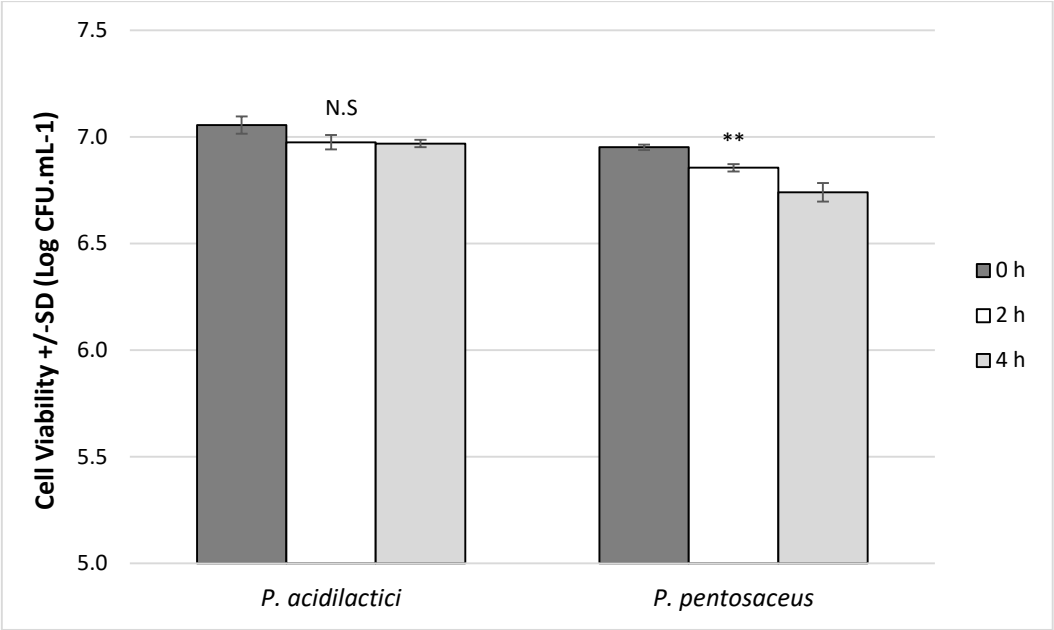


Figure 21. Cell viability of two antimicrobial compound-producing bacteria at 0 h and after 4 h exposure in simulated intestinal juice. All values are means with standard deviations of two replicates. *N. S* indicates no significant difference in the number of viable cells (log CFU.mL^{-1}) after 0, 2 and 4 h exposure. ** indicates significant difference in cell viability between 0 h, 2 h and 4 h exposure, $P<0.05$.

4.3.6. Adhesion capacity to intestinal mucus

The adhesion capacity of two examined LAB to the intestinal mucus of fish, is presented in Figure 22. In general, the figure shows that *P. pentosaceus* MA169 appeared to adhere better than *P. acidilactici*, indicated by higher calculated adhesion value: 20.9±5.2 % for *P. pentosaceus* MA169 and 10.3±3.9 % *P. acidilactici* respectively, t=4.66, df 14, $P<0.01$.

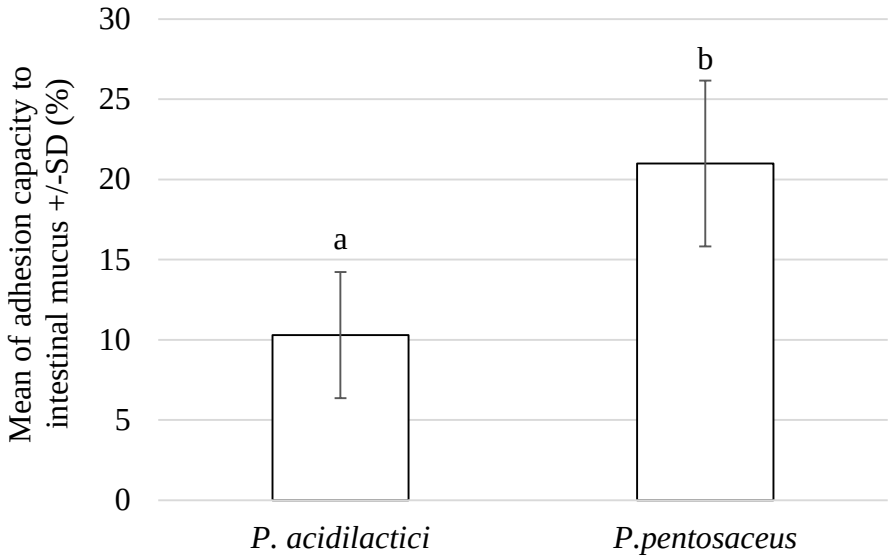


Figure 22. Adhesion capacity to fish intestinal mucus of *P. acidilactici* MA160 and *P. pentosaceus* MA169, presented in terms of the percentage of adhered bacteria in relation to the added dose. Values are means with standard deviation of 8 replicates. Different letters indicate significantly different adhesion ability ($P<0.01$).

4.3.7. Growth in intestinal mucus

P. acidilactici MA160 and *P. pentosaceus* MA169 were able to grow in intestinal mucus, Figure 23. In addition, the result showed that the growth of *P. pentosaceus* MA169 was significantly faster than the growth of *P. acidilactici* MA160 ($t=5$, df 5, $P=0.04$). Overall, the growth rate of *pentosaceus* MA169 in the intestinal mucus was almost twice that of *P. acidilactici* MA160. The results also indicated that *P. acidilactici* MA160 had a shorter lag phase (4 h) and generation period (17 h), compared to *P. pentosaceus* MA169, which had 12 h for the lag phase and 21 h for the generation period.

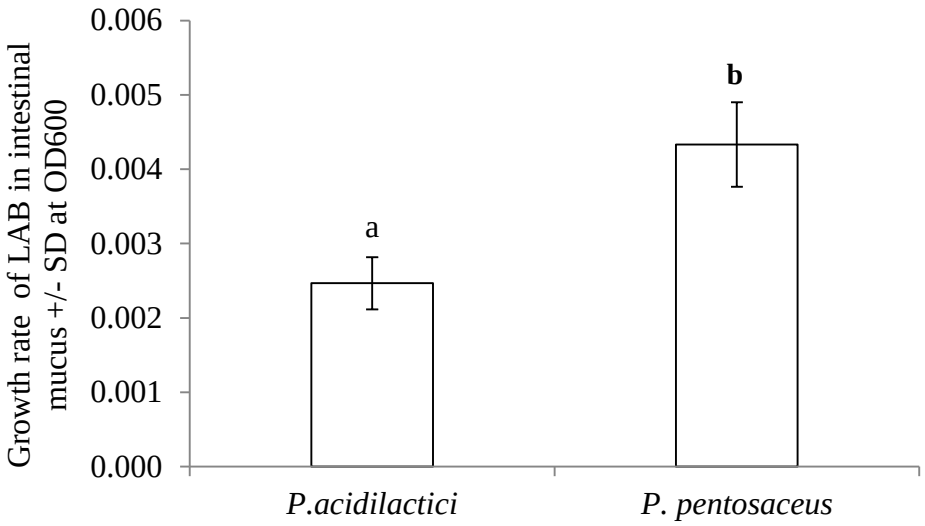


Figure 23**.** Growth rate (μ) of two antimicrobial compound-producing bacteria in intestinal mucus after 24 h incubation at room temperature. Growth was calculated based on the change of OD value at 600 nm in the exponential phase. Values were means of growth rates with standard deviation of 6 replicates.

4.3.8. Susceptibility to antibiotics

Both *P. acidilactici* MA160 and *P. pentosaceus* MA169 showed their susceptibility to chloramphenicol (10 µg), indicated by the formation of an inhibition zone around the antibiotic disk, Figure 24. This result suggested that all tested strains do not carry resistant genes to the antibiotic.

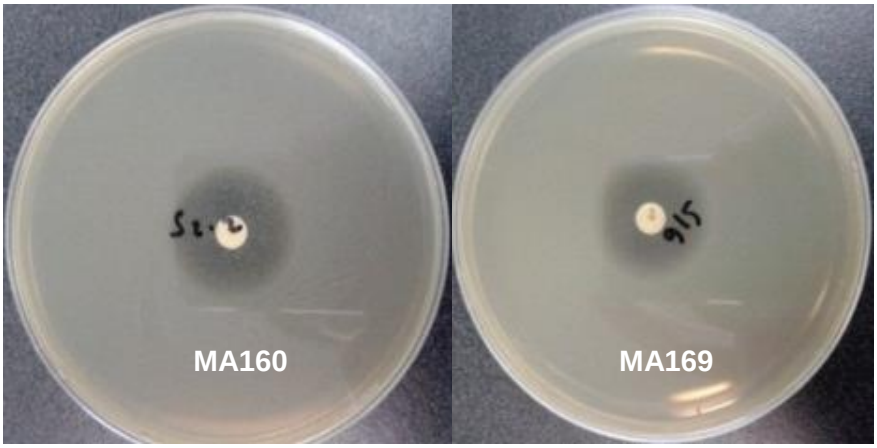


Figure 24. Clearance zone due to inhibition activity of chloramphenicol (10 µg) on probiont candidates. *P. acidilactici* MA160 and *P. pentosaceus* MA169.

4.4. Discussions

4.4.2. LAB antimicrobial-compound production

LAB isolated from aquatic animals are suggested to be better probionts for aquaculture industries, because they are well adapted to the conditions in aquatic animals (Fjellheim et al., 2010). This study screened 206 LAB isolated from the GITs of teleosts and molluscs for antagonistic activity against eight bacterial pathogens (seven aquatic pathogens and one foodborne pathogen frequently reported in seafood). The results showed that 22 LAB had inhibitory activity against one or more indicator pathogens. Based on 16S rDNA sequence, the antagonistic LAB belonged to six genera: *Carnobacterium* (2 strains), *Enterococcus* (12 strains), *Lactobacillus* (1), *Lactococcus* (1), *Leuconostoc* (1) and *Pediococcus* (2 strains).

Carnobacterium

Two strains of genus *Carnobacterium* belonging to the species *C. divergens* and *C. maltaromaticum*, showed inhibitory activity against *A. hydrophila* and *V. harveyi*, respectively. Previously, there were few studies concerning the antimicrobial activity of these species against aquatic and foodborne pathogens. For instance, *C. divergens* has been reported to be antagonistic against *Clostridium perfringens* (Jozefiak et al., 2012), *L. monocytogenes* (Brillet et al., 2005), *V. anguillarum, V. viscous* (Ringo et al., 2002) and also *A. hydrophila* (Ringø, 1999). In addition, *C. maltaromaticum* has been described with antagonistic activity against *A. salmonicida* and *Y. ruckeri* (Kim and Austin, 2006), and *A. hydrophila, Streptococcus inniae* and *V. anguillarum* (Kim and Austin, 1961). These previous studies suggested that the *Carnobacterium* appeared to have broad ranges of antimicrobial compounds. Contrarily, the present study indicated a narrow inhibitory activity of both carnobacteria.

Enterococcus

sixty-eight percent (15/22) of the antagonistic LAB belonged to Enterococcus: *Enterococcus* sp. (1 strain), *E. durans* (3 strains), *E. faecium* (5 strains), *E. faecalis* (1 strain), *E. thailandicus* (3 strains), *E. malodoratus* (1 strain) and *E. raffinossus* (1 strain). Many studies have previously reported the production of antimicrobial compounds from species reported in this study, Table 16**.** However, to the author's knowledge, there were no studies which had reported the antimicrobial production by either *E. malodoratus* or *E. raffinosus*. In fact, both species have been described as pathogenic and spoilage bacteria (Moellering Jr, 1992; Freyaldenhoven et al., 2005). Thus, investigating the toxicity of these bacteria are strongly recommended for further studies.

Table 16. Antagonistic enterococci against several bacterial pathogens

No	Enterococcus	Targeted Pathogens	Reference
1	*E. faecium*	*L. monocytogenes*	Viera et al. (2005)
		Salmonella sp.	Audisio et al. (1999)
		Edwardsiella tarda	Chang and Liu (2002)
		A. hydrophila	Gopalakannan and Arul (2011)
		V. cholerae	Simonetta et al. (1997)
		V. harveyi	Swain et al. (2009)
2	*E. durans*	*L. monocytogenes, E. coli,*	Pieniz et al. (2014)
		S. typhimurium, A. hydrophila	
3	*E. thailandicus*	*L. monocytogenes*	Jaouani et al. (2014)
4	*E. faecalis*	*L. monocytogenes*	Galvez et al. (1998)
		S. aureus	

Lactobacillus

The present study observed that one strain of *Lactobacillus,* identified as *Lb. farraginis*, had inhibitory activity against two common aquatic pathogens, *V. anguillarum* and *V. harveyi*. There have been only a few studies reported about the isolation of this species, including from distilled Sochu residue (Endo and Okada, 2007), mezal fermentation of

Agave salmina (Escalante-Minakata et al., 2008) and a greenhouse soil (Seo et al., 2012). Among the studies, only one reported antagonistic activity of *Lb. farraginis*, which was used as a biocontrol agent against a root-knot nematode in oriental melon (Endo and Okada, 2007).

Leuconostoc and ***Lactococcus***

Leuc. mesenteroides and *Lact. lactis* subsp. *lactis* appears to be quite common as probionts, and this present study confirmed the antagonism produced by these species against *V. alginolyticus*. Previously, *Leuc. mesenteroides* has been reported to be bactericidal against *L. monocytogenes* (Daba et al., 1991; Mataragas et al., 2003; Trias et al., 2008), *A. salmonicida* (Balcazar et al., 2009), and *P. aeruginosa* and *S. putrefaciens* (Allameh et al., 2012). *Lac. lactis* was also reported to be antagonistic against several bacterial pathogens, including *A. hydrophila* (Zhou et al., 2010), *L. monocytogenes* (Benkerroum et al., 2000), and *C. perfringens.* and *S. aureus (Spelhaug and Harlander, 1989)*. In general, this present study suggested that both LAB had narrower activity spectra against bacterial pathogens compared to previous studies.

Pediococcus

P. acidilactici and *P. pentosaceus* had been reported to have a broad spectrum of antimicrobial activity against several pathogens. *P. acidilactici*, for instance, was antagonistic toward *V. anguillarum* (Harper et al., 2011), *Streptococcus inniae* (Hoseinifar Seyed et al., 2016), *Tenacibaculum maritimum* and *V. splendidus* (Munoz-Atienza et al., 2014). Meanwhile, *P. pentosaceus* had been documented to inhibit the growth of *V. anguillarum* (Huang et al., 2014), *L. monocytogenes* (Jang et al., 2014) and *Photobacterium damselae* subsp. *piscicida* (Xing et al., 2013). In fact, *Pediococcus acidilactici* is the only authorised/registered probiotic for aquaculture in the EU (https://en.engormix.com). This

present study confirmed the antimicrobial production by these species, as indicated by the inhibitory activity against several indicator pathogens: *A. hydrophila*, *V. alginolyticus*, *V. harveyi*, *V. proteolyticus*, and *Y. ruckeri*. Other studies showed that the supplementation these LAB could improve the disease resistance of cultured aquatic species such as red tilapia (Ferguson et al., 2010), turbot (Villamil et al., 2010), rainbow trout (Hoseinifar Seyed et al., 2016), shrimp (Castex et al., 2009) and grouper (Huang et al., 2014). Thus, the incorporation of these antagonistic LAB could be examined as an alternative means to increase animal resistance against bacterial pathogens.

4.4.3. Antimicrobial compounds and inhibitory mechanism

There are several antimicrobial compounds which have been commonly reported from LAB, including organic acids (Goncalves et al., 1997; Vazquez et al., 2005), bacteriolytic enzymes, hydrogen peroxide (Verschuere et al., 2000a) and bacteriocins (Verschuere et al., 2000a; Ringo, 2008; Yanagida et al., 2008; Lin et al., 2013). The antagonistic activity of organic acids has been generally associated with a decrease in the pH values of the culture medium (De Vuyst and Vandamme, 1994; Presser et al., 1997), which was confirmed in this present study. In addition, the inhibitory activity which remained after the pH of CFS was neutralized indicated that there were other antimicrobial substances, such as bacteriocin-like substances.

4.4.3.1. Organic acids

Several studies have reported the antagonistic activity of organic acids towards Gram-negative pathogens such as *Echericia coli*, *Pseudomonas aeruginosa*, *Streptococcus enterica*, *V. anguillarum* and *A. salmonicida* (Alakomi et al., 2000; Vazquez et al., 2005). The antimicrobial activity of organic acids was described by their ability to disintegrate the cell wall and cross over pathogens' membranes, dissociating, and acidifying internal pathogens, resulting in reduced intracellular pH (Goncalves et al., 1991; Akerberg et al.,

1998; Alakomi et al., 2000; Vazquez et al., 2005). These processes were finalized by a disruption of the pathogens' metabolic processes by promoting the expulsion of H^+ ions from the cell and causing the uncoupling of the Na^+ -K^+ (ATPase) pumps (Goncalves et al., 1997; Presser et al., 1997).

However, the antagonistic action of organic acids was highly pH dependent. In low pH, organic acids such as lactic acids tended to be present in an undissociated form, which is the toxic state (Cachon and Divies, 1994). Meanwhile, in pH 6.5 and above, the lactic acids were present in the dissociated form, which is the non-toxic state (Goncalves et al., 1997; Vazquez et al., 2005). This clue might explain the result of this present study in which unneutralized CFS (pH; 4.82 ± 0.39) showed stronger antagonistic activity towards the indicator pathogens compared to neutralized CFS (pH 6.5). Due to their antimicrobial properties, lactic acids have been considered as safe for use in natural preservation especially in food products (Alakomi et al., 2000).

$$\underset{\text{(undissociated form)}}{CH_3CH(OH)CO_2H} \leftrightarrows H^+ + \underset{\text{(dissociated form)}}{CH_3CH(OH)CO_2} \quad Ka = 1.38 \times 10^{-4}$$

Equation 1. Toxic and non-toxic states of lactic acids

In addition, the antimicrobial activity of the CFS remained after the pH was neutralized (~6.8) with 1 M NaOH, which may suggest the presence of other antimicrobial compounds, including hydrogen peroxides and bacteriocin-like substances. However, the formation of hydrogen peroxides was prevented since the LAB was cultured anaerobically. Therefore, the other possibility would be bacteriocin-like substances.

4.4.3.2. Amplification of a fragment gene encoding pediocin-like substances

Bacteriocins are biologically active proteins with a bactericidal activity produced by certain bacteria as part of an antagonistic weapon against closely-related bacteria (Riley and

Wertz, 2002). The interest in bacteriocins as an alternative replacement for antibiotic use in aquaculture has increased considerably in the last few decades. This study only could confirm that two pediococci (*P. acidilactici* MA160 and *P. pentosacesus* MA169) appeared to have Pediocin-PA1 like encoding gene. Since there was no positive control for pediocin PA-1 encoding gene, it was unclear whether the amplified gene was the targeted gene or a contaminant. However, based on the band size, the gene amplified from *P. pentosaceus* MA169 was the same size as previously detected by Todorov and Dicks (2009), ~ 1000 bp which may indicate it was the correct gene. However, the band size for Pediocin-like substance detected from *P. acidilactici* was ~ 500 bp, which is 300 bp shorter than reported by Todorov and Dicks (2009). Perhaps it was a contaminant, or the differences might be due to different bacterial strains or other types of bacteriocins that happened to be quite close to the targeted bacteriocins. However, in order to get more comprehensive results, these bands need to be sequenced. In addition, the expression of these genes also requires further study by treating the CFS with proteinase K.

4.4.4. Capacity to colonize fish GITs

Besides having the capacity to produce antimicrobial compounds against pathogens, probiotic candidates should be able to colonize the GITs of their host (Nikoskelainen et al., 2001b; Fernandez et al., 2003; Vine et al., 2004; Merrifield et al., 2010a; Harper et al., 2011; Geraylou et al., 2014). Some parameters which can be used for evaluating the colonization capacity of probiotic candidates are: (1) survival rate in simulated stomach juice, (2) survival in simulated intestinal juice, (3) adhesion capacity and (4) the ability to grow in fish intestinal mucus (Nikoskelainen et al., 2001b; Geraylou et al., 2014). This present study investigated the capacity of *P. acidilactici* MA160 and *P. pentosaceus* MA169 which had broad spectrum of inhibitory activity against *5* vibrios, to colonize the GITs of aquatic species. The two LAB had good tolerance in both SSJ and SIJ. This result suggests that these LAB were resistant

to the low pH of the stomach (Harris et al., 1998) and to bile salt in the intestine (Geraylou et al., 2014). This result also confirmed the good tolerance of LAB to low pH (Furr. M, 2013) and bile salts (Geraylou et al., 2014). The ability to tolerate bile salt might be due to the pediococci have the ability to produce bile salt hydrolase or a coating of exopolysaccharides that could protect them from bile salts (Geraylou et al., 2014). However, further assays are still needed to confirm the presence of those protective agents and their protective mechanisms.

Furthermore, these pediococci were able to adhere and grow in fish intestinal mucus. The adhesion rate of *P. pentosaceus* MA169 and *P. acidilactici* MA160 to intestinal mucus was 21 % and 10 %, respectively. The adhesion rate was higher compared to adhesion capacity of bacterial strains reported in previous studies (Nikoskelainen et al., 2001b; Geraylou et al., 2014). The adhesion capacity of *Lactobacillus casei* Shirota and *Lb. rhamnosus* LC 705 isolated from humans, on the intestinal mucus of rainbow trout were only ±1.1% and 0.6 %, respectively (Nikoskelainen et al., 2001b). In addition, Geraylou et al. (2014) reported that the adhesion capacity of *Lact. lactis* and *B. circulans* in intestinal mucus of Siberian sturgeon (*Acipenser baerii*) was only 6 % and 4 %, respectively. As adhesion ability is regarded as one of the most important criteria for new probiotic candidates (Robertson et al., 2000), this present result suggests that endogenous microbial communities seem to be a good source from which probiotic candidates could be isolated. The better adhesion ability might be due to the presence of specific receptors for intestinal mucus or to carbohydrate-specific molecules of the LAB strains' surfaces which act as mediators for adhesion to intestinal mucus (Servin and Coconnier, 2003; Rawls et al., 2004; Geraylou et al., 2014). As both pediococci were isolated from GITs of fish, this result confirms what has been suggested by Fjellheim et al. (2010) in which probiotics isolated from aquaculture animals would have better adhesion and colonization capacity than probionts from terrestrial

organisms. These results suggest that *P. acidilactici* MA160 and *P. pentosaceus* MA169 are potential probiotic candidates in aquaculture species. However, colonization capacity of these pediococci on intestinal mucus should be further studied by *in vivo*.

4.5. Conclusion

Twenty-two LAB showed inhibitory activity against at least one of eight bacterial pathogens. The antagonistic compounds appeared to be due to organic acids and bacteriocin-like inhibitory substances (BLIS). Two LAB were selected for further *in vitro* studies due to displaying the broadest antimicrobial spectra against five vibrios. The results showed that the 2 LAB had the potential capacity to colonize the GITs of aquatic species, indicated by high resistant to low pH, bile salts and gastric enzymes (trypsin and pepsin) in the simulated stomach and intestinal juice, and having a good capacity to adhere and grow in intestinal mucus. These results suggest that these LAB are potential probionts in aquaculture. However, further *in vivo* studies need to be conducted for more comprehensive results.

Chapter 5 : The Effect of Probiotic Supplementation on The Growth and Survival Rates of Abalone

5.1. Introduction

The slow growth of abalone is still regarded as a major constraint in abalone aquaculture industries. Hahn (1989) reported that the typical growth rate of *H. asinina* in the natural environment was 20-30 mm year^{-1} of shell length, therefore it requires 2-5 years to reach a marketable size of 80 mm. Many studies have described that the low growth of cultivated animals is frequently associated with low digestibility and absorption of diets, indicated by high values of FCR such as 7 for *Haliotis asinina* (Bautista-Teruel and Millamena, 1999). The poor digestibility of diet is caused by the low amount or activity of digestive enzymes in the animals' intestinal tracts (Viveros et al., 1994; Lemieux et al., 1999; Kolkovski, 2001; Emiola et al., 2009; Lee and Lawrence, 2009). Other studies have described that macroalgae, which is the most preferred natural diet of abalone, were very difficult to digest due to several compounds, including cellulose as the most common skeletal polymer (Kregar 1962) and alginate (Gomez-Pinchetti and Garcia-Reina, 1993). Sawabe et al. (1995) reported that the alginate content in macroalgae consisted of polyguluronate (polyG) block and polymannuronate (polyM) block, and the endogenous alginate lyase secreted by abalone was only able to break down polyM-block but not polyG-block. Therefore, alternative approaches to improve digestibility and absorption of the nutrient contents in macroalgae to increase the growth of abalone have been continuously investigated.

Based on previous studies from other aquatic animals, there are some possibilities which can be used to increase feed digestibility in cultivated animals, including: (1) increasing the amount of digestive enzymes as a tool to break down feed particles (Lemieux et al., 1999), and (2) enhancing the solubility and absorption of nutrients in the GITs of aquatic species (Merrifield et al., 2010b). These two approaches have been supported by several studies, in which several bacterial species associated with the GIT of aquatic abalone

have been identified as participating in feed digestion, due to their capacity to produce digestive enzymes (Gomez-Pinchetti and Garcia-Reina, 1993; Erasmus et al., 1997). In addition, it has been reported that lowering the pH of the GIT enhanced solubility and absorption of nutrients (Merrifield et al., 2010b). Given these facts, the supplementation of digestive enzymes and acid-producing bacteria have been considered as potential ways to increase the growth of abalone. This approach is also becoming increasingly popular due to the surging demand for environmentally friendly aquaculture products.

Several studies have confirmed that supplementation of probiotic candidates enhanced growth rates and disease resistance in farmed abalone (Macey and Coyne, 2005; ten Doeschate and Coyne, 2008; Iehata et al., 2010; Silva-Aciares et al., 2011; Jiang et al., 2013a; Hadi et al., 2014). The supplementation of protease-producing *Vibrio midae* via diets increased protein digestibility and improved the growth rate of juvenile *Haliotis midae* (Macey and Coyne, 2005). Similarly, Hadi et al. (2014) observed that the incorporation of protease-producing *Exiguobacterium* JHEb1 and alginate lyase-producing *Vibrio* JH1 improved the feed digestibility and growth rate of *H. iris*. Similarly, alginate lyase-producing *Pseudoalteromonas* sp. strain C4 incorporated in kelp showed growth improvement in *H. midae* (ten Doeschate and Coyne, 2008). The supplementation of organic acid-producing *Enterococcus* sp. increased not only growth, but also the survival rates of *H. iris* (Hadi et al., 2014). This may be explained by the antagonistic activity of organic acids, generally produced by members of LAB group, including genus *Enterococcus* (Goncalves et al., 1991; De Vuyst and Vandamme, 1994; Presser et al., 1997; Akerberg et al., 1998; Alakomi et al., 2000; Vazquez et al., 2005). It has been proposed that the administration of probiotic strains, stimulating the innate immune system of abalone, resulted in significantly increased survival rates of juvenile *H. discuss hannai* against *V. harveyi* (Jiang et al., 2013b). However, to the

author's knowledge, there are no studies which have applied probionts to increase the growth and survival rates of tropical abalone, *H. asinina*.

Therefore, the study described here evaluated the supplementation of three probiotic candidates isolated and described previously in chapters 2 - 4 (alginate lyase-producing *Enterobacter ludwigii*, protease-producing *Bacillus amyloliquefaciens* subsp. *plantarum* and acid-producing *Pediococcus acidilactici*) on the growth performances and survival rates of abalone. The isolation and characterization of these probiotic candidates is described previously in chapters 2 - 4 and the bacteria were an alginate lyase-producing *Enterobacter ludwigii*, a protease-producing *Bacillus amyloliquefaciens* subsp. *plantarum* and an acid-producing *Pediococcus acidilactici*). Two hypotheses were tested: (1) the growth of juvenile abalone receiving two digestive enzyme-producing bacteria (2P)-supplemented diet is higher than the abalone without the bacterial supplementation, and (2) the growth rate and survival of juvenile abalone receiving three probiotic candidates (3P)- supplemented diet are higher than abalone receiving either a 2P-supplemented diet or without bacterial supplementation (control).

5.2. Materials and Methods

5.2.1. Probiotic strains and culture media

Three probiotic candidates used in this present study were: *E. ludwigii*, *B. amyloliquefaciens* subsp. *plantarum* (described in chapter 3), and *P. acidilactici* MA160 (description in chapter 4). These 3 probiotic candidates were chosen based on the result of a preliminary study in which *E. ludwigii* gave the highest growth on hybrid abalone, followed *B. amyloliquefaciens* subsp. *plantarum*. In addition, another bacterial strain (*P. acidilactici* MA160) was added as another treatment due to its ability to produce organic acids and other antimicrobial

compounds, as well as species which has been authorised/registered probiotic for aquaculture in the European Union (EU).

The three bacterial strains were cultured in either 5 mL TSB or MRS broth with seawater and incubated at room temperature aerobically. The purity of each bacterial strain was checked by streaking onto either TSA or MRS agar. A single well-separated colony was picked up and sub-cultured in 5 mL broth media and incubated for 24 h aerobically. Afterward, 5mL culture was inoculated into 500 mL broth media for upscaling. After 24 h incubation, the broth culture was diluted in 5L sterilized seawater (OD_{600}: 0.15) in which the *Gracillaria* sp. was immersed.

5.2.1. Animal sources

A total of 225 juvenile abalone, *H. asinina,* with mean weight and length of 0.51-0.53 g and 15-16 mm respectively were used in this present study. The juvenile abalone were harvested from a natural spawning in a hatchery of the Marine Development Aquaculture Centre, West-Nusa Tenggara, Indonesia, Figure 25.

Figure 25. Juvenile abalone, *H. asinina,* attached on a hemispherical PVC shelter**.**

5.2.2. Experimental rearing condition

The abalone were placed in a floating basket and reared in nine 40L-plastic tanks and supplied with fresh filtered seawater and continuously aerated, Figure 26. Each rearing tank had 25 abalone and each abalone was tagged with a unique number (1-25) to track individual growth. Then, the abalone were acclimatized for one week prior to the feeding experiment, and fed with the unsupplemented macroalgae, *Gracillaria* sp. at 45 % of total body weight (BW) day^{-1}. An artificial shelter of a hemispherical PVC was provided in each tank. During the rearing period, the physicochemical parameters of the rearing water were kept within a range of optimal conditions for the tropical juvenile abalone; salinity (28-32ppt), temperature, 27-31 ^{o}C (Ganmanee et al., 2010), DO (5.0-5.6 mg L^{-}), pH (8.3-8.4) (Bautista-Teruel and Millamena, 1999), ammonia <0.46 mg L^{-1} and nitrite < 0.5 mg L^{-1} (Weirich and Riche, 2006) for the duration of the experiment. The seawater used for this experiment was filtered through a 0.2 μm filter prior to use. Rearing water was replenished 100 % daily before feeding.

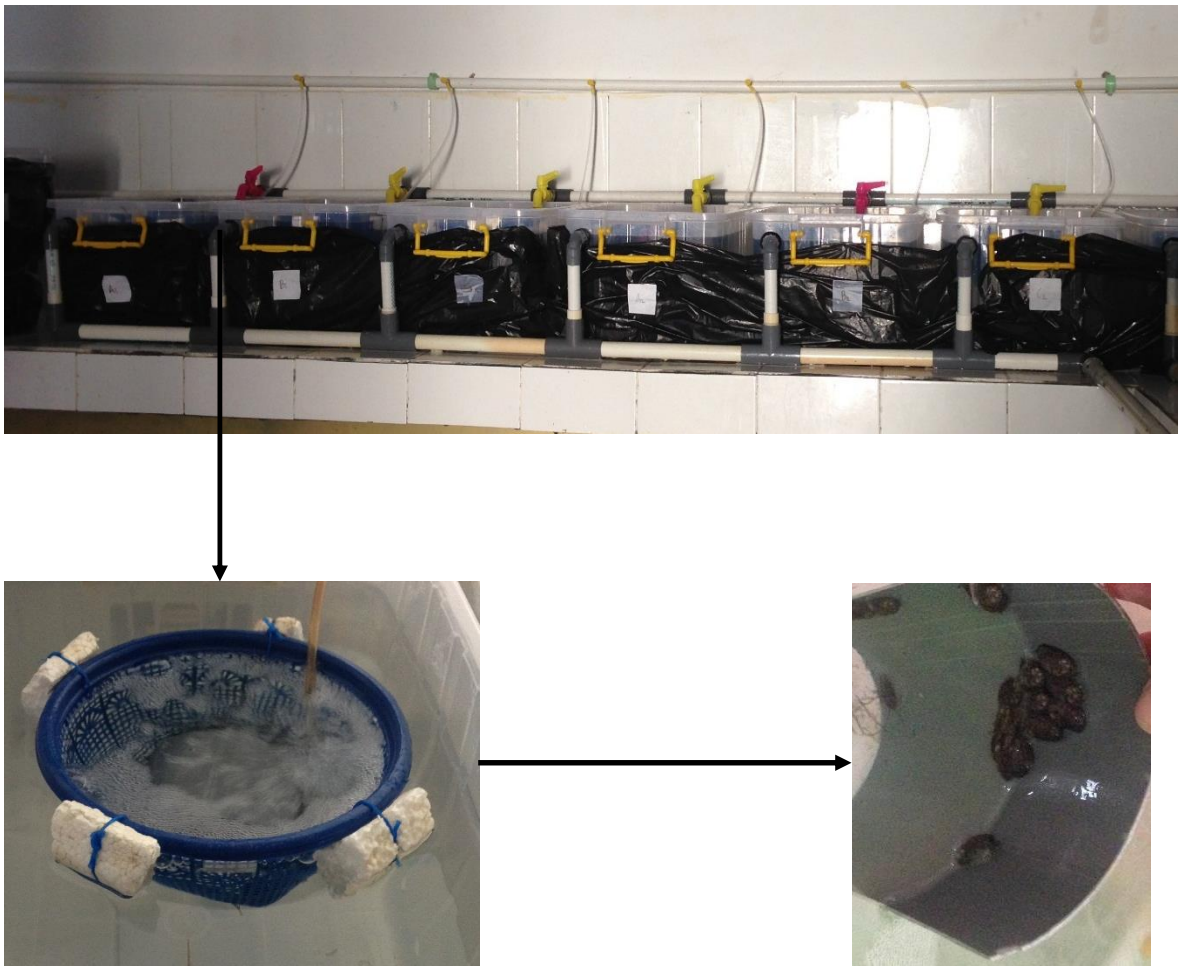


Figure 26. Juvenile abalone in floating baskets within a flow-through rearing system.

5.2.3. Experimental setup

The feeding experiment was divided into three experimental groups: one group received diets supplemented with two probiotic candidates (*E. ludwigii* and *B. amyloliquefaciens* subsp. *plantarum*), one group was fed on diets supplemented with three probiotic candidates (*E. ludwigii*, *B. amyloliquefaciens* subsp. *plantarum* and *P. acidilactici*) and the other group received the unsupplemented diet, *Gracillaria* sp., as a control, Figure 27. Each treatment had three replicates of rearing tanks, and each tank had 25 juvenile abalone.

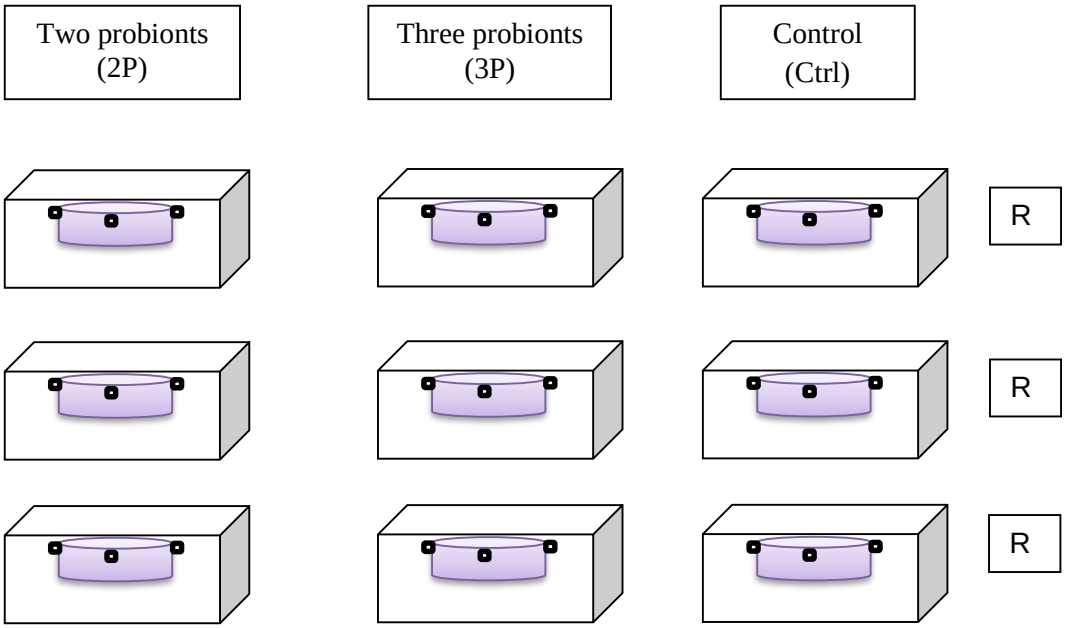


Figure 27**.** Experimental setup and rearing tanks. R (1-3) are the replicate tanks**.**

5.2.4. Feed preparation

The supplementation of probiotic candidates was conducted via seaweed, *Gracillaria* sp. as a vector according to a modified protocol of Silva-Aciares et al. (2011). In brief, fresh *Gracillaria* sp. obtained from seaweed farmers was washed with filtered seawater to remove epiphytic organisms. The seaweed was then placed in a 40-L plastic bucket containing filtered seawater, which was previously disinfected with chlorine at 0.025 g L^{-1}. After 1 h immersion, the chlorine-seawater mixture was neutralized by adding sodium thiosulfate to reach a concentration of 0.075 g L^{-1}. Subsequently, the disinfected seaweed was moved and immersed in a 5L solution of either two probiotic candidates (2P), or three probiotic candidates (3P) with the cell concentration of ~7 Log CFU mL^{-1}, or in sterilized seawater for the control. After 24 h immersion with constant aeration, the seaweed was taken out and used to feed the abalone, Figure 28. The preconditioning of *Gracillaria* sp. with the probionts or sterilized seawater was conducted every three days.

Figure 28. Macroalgae, *Gracillaria* sp., after immersion in probiotic solution.

5.2.5. Feeding experiment

The feeding experiment was conducted over 62 days at Marine Aquaculture Development Center, West-Nusa Tenggara, Indonesia. Firstly, the juvenile abalone were starved for three days prior to the start of the trial to empty their guts. The abalone were then fed with either one of the three diet groups (2P-impregnated diet, 3P-impregnated diet or unsupplemented diet) – see section 5.2.5 for preparation details. The abalone were fed *ad libitum* feed, ~135 %BW 3 $days^{-1}$.

5.2.6. Observed parameters

During the experiment, there were several parameters which were observed: individual weight and length, feed intake, total viable count for bacteria in the rearing water and in the *Gracillaria* sp., physicochemical parameters of water quality (temperature, salinity, pH, dissolved oxygen, ammonia, and nitrite), mortality and proximate analysis of feces and the diet.

a) Weight and length

The wet weight and shell length of the abalone were measured at the beginning and the end of the experimental periods. For the weight, the abalone were

detached carefully from either the artificial shelter or the surface of the floating bucket using a thin flexible scalpel. The detached abalone were then dried using a piece of tissue and then weighed to the nearest 0.01 g difference. Each animal was photographed, and the shell length measurement was taken on the longest axis to the nearest 0.01 mm using software ImageJ, Figure 29. Then total weight gain (WG), total shell length gain (LG), and the specific growth rate in terms of weight (SGR_w) and length (SGR_L) were calculated according to the following formula (Mulvaney et al., 2013).

$$WG = W_t - W_0$$

$$LG = L_t - L_0$$

$$DGw = 1000\ (\frac{W_t - W_0}{t})$$

$$SGRw = 100\ (\frac{log_e\left(\frac{W_t}{W_0}\right)}{t})$$

$$SGR_L = 100\ (\frac{log_e\left(\frac{L_t}{L_0}\right)}{t})$$

$$FCR = \frac{Total\ feed\ consumed\ (g)}{Total\ weight\ gained\ (g)}$$

Where: SGR_w= Specific growth rate in terms of wet weight (% body weight. day^{-1}); SGR_L= Specific growth rate in terms of shell length (% body length. day^{-1}); W_t = final weight (g); W_0 = initial weight (g); L_t= final shell length; L_0 = initial shell length; t= time (day); WG= weight gain; LG= shell-length gain. Daily growth in terms of weight (DG_W) and length (DG_L), and FCR = feed conversion ratio.

Figure 29. Measurement of shell length

b). Feed intake

The amount of diet given was weighed before being fed to the abalone. Then after 16 h feeding time, the uneaten diet was dried by placing it on a piece of tissue paper and weighed. This was performed every three days. Feed intake was then calculated by subtracting the final weight of the diet from the initial weight of the diet, Figure 30.

Figure 30. Drying and weighing of uneaten seaweed.

c). Total viable count (TVC)

The total count of viable heterotrophic bacteria in the experimental tanks was performed weekly using Plate Count Agar (PCA) to monitor bacterial concentrations in both the rearing water and in the diet, according to a protocol developed by Sawabe et al. (1995). In brief, 10 mL of rearing water from each experimental tank was

serially decimally diluted (10^{-1} to 10^{-6}) in 0.85 % saline solution (SS), and 100 µL of each dilution was spread on the surface of duplicate PCA plates (PCA; EMD Millipore 105463). The plates were incubated at 29ºC aerobically. After 24-48 h, the number of bacterial colonies was enumerated.

The total bacterial count attached to the abalone diet, *Gracillaria* sp was also evaluated weekly according to a modified protocol of Silva-Aciares et al. (2011). Briefly, 2-5 g of seaweed from each experimental group was firstly homogenized with a mortar, (Figure 31). The homogenized seaweed was then serially decimally diluted (10^{-1}-10^{-6}) in 0.85% NSS. Afterwards, 100 µL from each serial dilution was pipetted out and spread onto duplicates of PCA plates. After 24-48 h incubation at 29 ºC aerobically, the number of colonies on each plate was enumerated.

Figure 31. Homogenization of seaweed using a mortar.

d). Mortality

Mortality was checked daily and any dead animals were removed from the tank during the trial and replaced with new abalone. However, the new abalone were not included in any analysis, but were kept in the tank simply to maintain a constant density.

e). Proximate analysis

Proximate analysis including ash and crude protein content in the *Gracillaria* sp. and abalone feces (Figure 32**)** was conducted using the standard protocols of the Laboratory of Analytic Chemistry, University of Mataram, Indonesia. In brief, 3-5 g of seaweed or faeces sample was put into an oven with a temperature of 100-102 ^{o}C for 1-3 h. The sample was then removed and placed into a desiccator for 60 min, and then weighed. The known amount of dried sample was placed in a porcelain crucible and placed in a muffle furnace at 550 ^{o}C. After 1 h, the sample was taken out and placed directly in a desiccator. The sample was weighed and put again into the muffle furnace until a constant weight was achieved. Finally, the ash content of the sample was calculated according to this formula:

$$\text{ash content } \left(\frac{\text{g}}{\text{kg}}\right) = \left(\frac{G_d - G_e}{G_s - G_e}\right) \text{ x } 1000$$

Where:

G_d= the lowest weight of the crucible after ashing

G_e= the weight of the empty crucible, and

G_s= the weight of the crucible and sample

Meanwhile, the crude protein (CP) content was analyzed using a Kjeldahl method. In brief, 50-100 mg of dried feces/seaweed was weighed and mixed with 3-10 mL of 0.01-0.02 N hydrochloric acid (HCl). Afterwards, the solution was placed into a digestion flask, and boiled for 1-1.5 h in the presence of 1.9±0.1 g potassium sulfate (K_2SO_4), 40±10 mg mercury oxide (HgO) and 2±0.1 mL sulfuric acid (H_2SO_4). Water was added carefully to cool it down. After cooling, the solution in the digestion flask was transferred into a distillation apparatus. A solution of 5ml of boric acid (H_2BO_3) and 4 drops of an indicator (red methyl in 0.2 % alcohol) was prepared in a 125-mL Erlenmeyer. Then, the Erlenmeyer was placed under the

condenser of a distillation apparatus in such a way that the end of the condenser dipped into the solution. A total of 8-10 mL of NaOH-$Na_2S_2O_3$ was added to the funnel of the apparatus and the alkali was run into the distillation chamber. Approximately 15 mL of condensate was distillated in an Erlenmeyer and the rinsed condenser by adding a few drops of mix indicator to the distillate in the same Erlenmeyer. The Erlenmeyer content was diluted to 50 mL and titrated with HCL 0.02 to a violet endpoint. Then, the crude protein content was calculated according to the following formula:

$$N\ (\%) = HCL - mL\ blank \text{ x } Normality \text{ x } 14.007 \text{ x } \frac{100}{mg\ sample}$$

$$CP\ (\%) = \%\ N \text{ x } Conversion\ factor\ (6.25)$$

Where:
N = Nitrogen content of samples, and
CP = Crude protein content.

Figure 32. Abalone faeces collected from each rearing tank

5.2.7. Data analysis

The collected data for biochemical analyzes, growth (animal weight and shell length) and mortality were analyzed using an SPSS statistics program (IBM SPSS version 20). The data were firstly tested for normality using Kolmogorov-Smirnov, and variance tests using

Levene's test. Afterward, One-way analysis of variance (ANOVA) and Tukey post hoc tests were used to determine significant differences between the sample means at $p < 0.05$. In addition, survival rate was analyzed using the Kaplan-Meier test.

5.3. Results

5.3.1. Growth rate

The overall growth parameters of juvenile abalone during the 62-day feeding trials in each treatment are presented in Table 17 and 18. At the beginning, the initial mean weight of juvenile abalone was 0.51 – 0.53 g, which was not significantly different among the treatments and the control, $P<0.05$. After the 62-day feeding trial, the average final weights were 1.59 g, 1.48 g and 1.36 g for abalone receiving a diet supplemented with 2P and 3P, and the unsupplemented diet, respectively. In general, the highest final weight was recorded by those abalone receiving 2P; 0.11 g higher than abalone receiving 3P and significantly higher by 0. 23 g compared to the control group. In addition, the mean weight gain (WG), FCR and specific growth rate (SGR) calculated for each treatment were significantly different among the treatment groups, ($P<0.05$). Juvenile abalone fed on the 2P-supplemented diet recorded better WG, FCR, and SGR_W, followed by the abalone receiving the 3P-supplemented diet or the unsupplemented diet (control), (all $P<0.05$). However, those abalone receiving the 3P-supplemented diet recorded no significant difference in WG and FCR compared to the abalone in the control.

Table 17. Growth of juvenile abalone, *H. asinina*, receiving probiotic supplementation in terms of weight.

No	Observed variables	Treatments		
		Ctrl	**2P**	**3P**
1	Initial Weight (g)	0.53 ± 0.06^{a}	0.51 ± 0.03^{a}	0.51 ± 0.03^{a}
2	Final Weight (g)	1.36 ± 0.15^{a}	1.59 ± 0.08^{b}	1.48 ± 0.12^{a}
3	Total Weight Gain (g/abalone)	0.82 ± 0.10^{a}	1.07 ± 0.08^{b}	$0.96\pm0.11^{a,b}$
4	Total Feed intake (g/abalone)	10.36 ± 0.83^{a}	10.03 ± 0.52^{a}	10.96 ± 0.57^{a}
5	FCR	12.69 ± 0.69^{b}	9.37 ± 0.41^{a}	11.43 ± 0.54^{b}
6	DG_W (mg/day)	13.33 ± 1.94^{a}	17.28 ± 0.98^{b}	$15.49\pm1.94^{a,b}$
7	SGR_w (%BW/day)	1.46 ± 0.05^{a}	1.75 ± 0.07^{b}	1.62 ± 0.02^{b}

a:b = values are means of abalone weight with standard deviation of three replicates. values with the same superscript are not significantly different ($P<0.05$). Ctrl= no supplemented bacteria. 2P= *Gracillaria* sp. + *E. ludwigii* and *B. amyloliquefaciens* subsp. *plantarum*. 3P= *Gracillaria* sp. + (*E. ludwigii*, *B. amyloliquefaciens* subsp. *plantarum*, and *P. acidilactici*).

In terms of shell length, those abalone receiving the 2P supplemented diet had the highest final shell length, total shell-length gain and SGR_L, $P<0.05$, Table 19**.** Meanwhile, the supplementation of 3P had significant effect only on the SGR_L, but no significant difference in the final shell length or shell-length gain was recorded to the control ($P>0.05$), although the overall values were slightly higher compared to the abalone with the unsupplemented diet.

Table 18. Growth of juvenile abalone receiving probiotic supplementation, in terms of shell length.

No	Observed variables	Treatments		
		Ctrl	2P	3P
1	Initial shell length (mm)	16.28 ± 0.48^{a}	15.45 ± 0.94^{a}	15.23 ± 0.13^{a}
2	Final shell length (mm)	20.48 ± 0.79^{a}	22.92 ± 0.36^{b}	21.95 ± 0.67^{ab}
3	Total shell Gain (mm)	4.17 ± 0.50^{a}	7.37 ± 0.90^{b}	6.81 ± 0.65^{a}
4	SGR_L (%SL/day)	0.36 ± 0.03^{a}	0.62 ± 0.09^{b}	0.59 ± 0.05^{b}

Different letter indicates that there was a statistical difference, $P<0.05$

5.3.2. Survival rate

There was no significant difference in the survival rates of juvenile abalone receiving different probiotic candidates and the control, $\chi^2 = 3.391$, df =2, p = 0.18. Though, the average survival rates calculated for those juvenile abalone fed on microalgae supplemented with the three probiotic candidates (3P) appeared to be slightly higher than the abalone receiving the 2 probiotic candidates (2P) and followed by abalone receiving normal diet, with 82 %, 78 %, and 71 %, respectively, Figure 33.

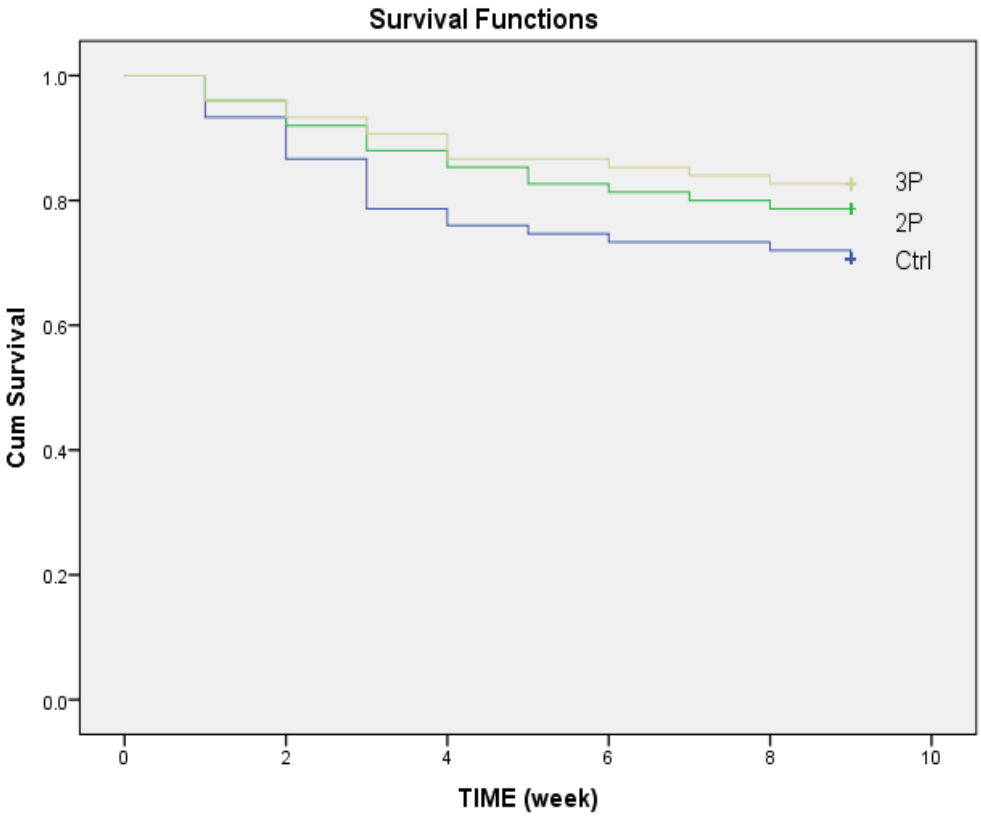


Figure 33. Survival rates of the abalone of different treatments

5.3.3. Total bacterial count

There was a significant difference in the average of bacterial count attached to the *Gracillaria* sp. among the three treatments during the experimental period, Figure 34. In general, total bacterial count in the probiotic-supplemented seaweed was significantly higher than TVC detected in the unsupplemented diet, all $P<0.05$. Meanwhile, the average TVC in both the 2P and 3P-supplemented diets were not significantly different, all $P>0.05$. The TVC detected from the macroalgae was also stable during the experiments, from 7 to 8 log CFU g^{-1} in the treated macroalgae and from 3 to 4 log CFU g^{-1} in the control. However, the TVC observed in the 2P-supplemented seaweed was counted to be higher in week 8, compared to the TVC in 3P-supplemented seaweed.

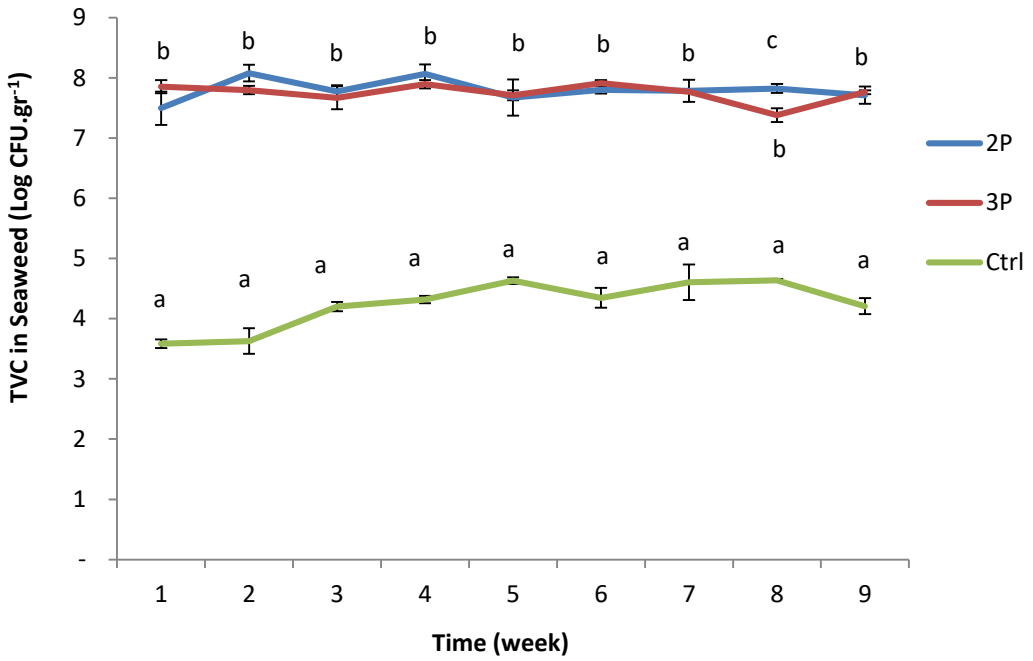


Figure 34. The changes of total viable count (TVC) of seaweed, *Glacillaria* sp. Different subscripts indicate significant difference in the means of total bacterial count, $P<0.05$.

The TVC in the rearing water was significantly higher in the treatment group compared to the control, Figure 35. In general, total bacterial counts from the rearing water of probiotic supplementations (2P and 3P) was 2 log higher than the amount of TVC in the rearing water of the control group. Meanwhile, the TVC was not significantly different among treatment groups (2P vs. 3P), except in week 7 in which the TVC of 3P-rearing water was significantly higher than the TVC of 2P rearing water, $P<0.05$. Overall, the TVC was kept stable in all treatments during the experimental period; ~2.4 log CFU mL^{-1} at rearing water of control and ~4.5 log CFU mL^{-1} at the rearing water of both treatment groups.

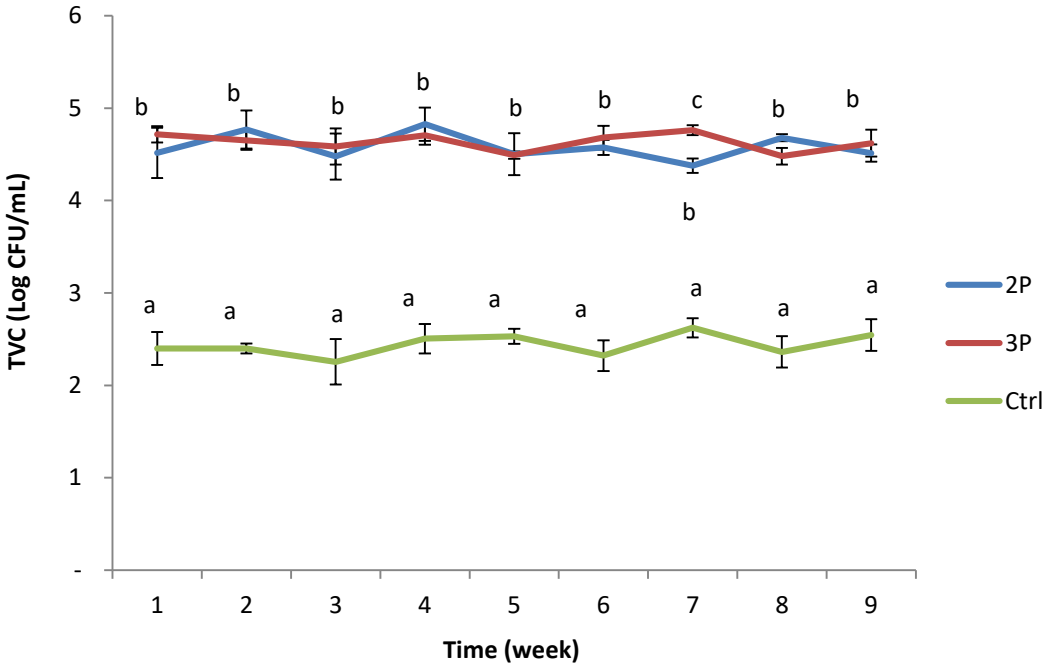


Figure 35. The changes of the total viable count (TVC) in the rearing water of all treatment groups. Different subscripts indicate significant difference in the means of total bacterial count, P<0.05.

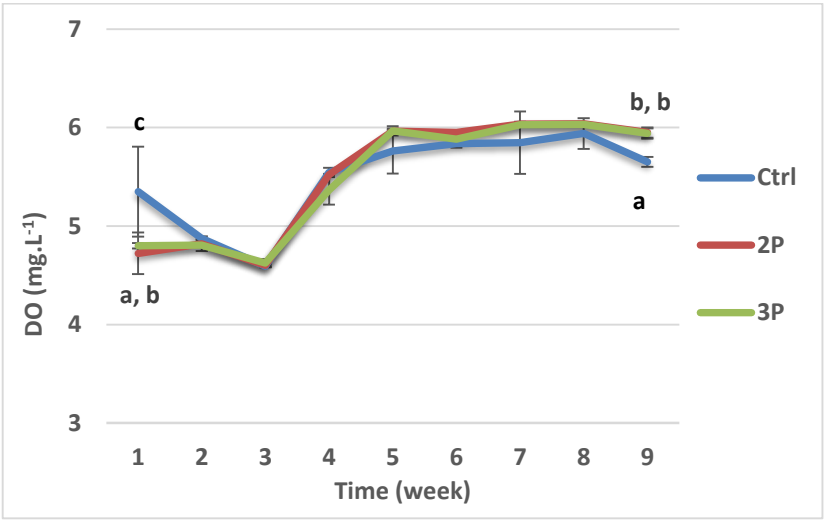
5.3.4. Water quality

All values of physicochemical parameters in rearing water were within safe level and appeared to be stable during the experimental period, Figure 36 & 37. Dissolved oxygen (DO) was recorded at values from 5 - 6 mg L^{-1}. All DO values were no significantly different from week 1 to week 8, all P>0.05. In week 9, the DO value was slightly lower at the rearing water of the control, P<0.05 (Figure 36a). Meanwhile, pH was measured to be around 8, and not significantly different among treatment groups during the experimental period, Figure 36b.

There was no significant difference in temperature and salinity of rearing water in all treatments, all P values <0.05. In general, the temperature was from 28 to 30 ^{o}C, and salinity

was 33-35 ppt, Figure 37a & b. Other parameters, such as ammonia, and nitrite, were also below toxic levels; ammonia (NH_3) < 0.25 and nitrite (NO_2) < 0.25 mg L^{-1}.

a



b

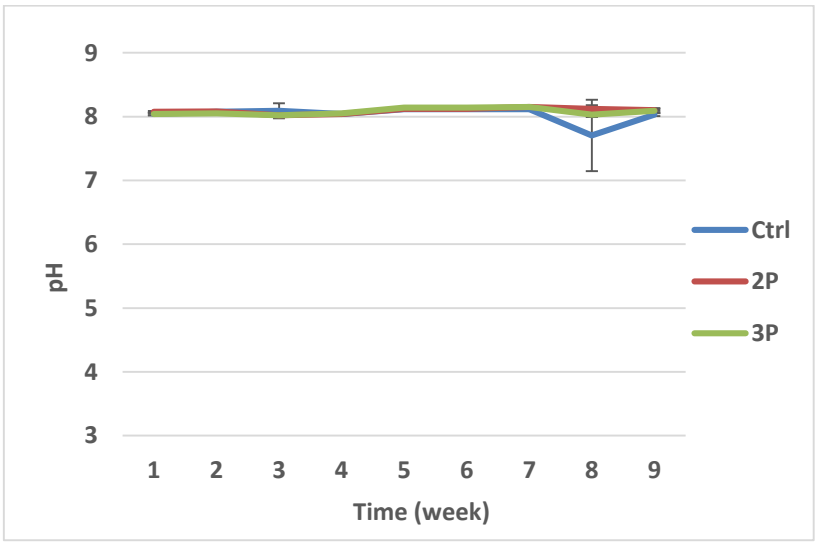
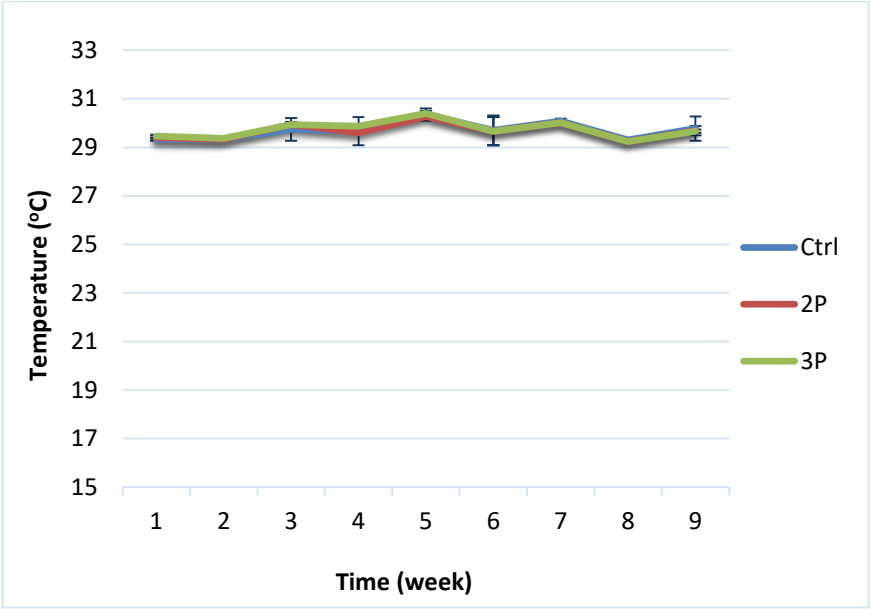


Figure 36. The changes in physicochemical parameters of the rearing water: (a) dissolved oxygen (DO), and (b) pH. Different subscripts indicate significant difference at P<0.05.

a



b

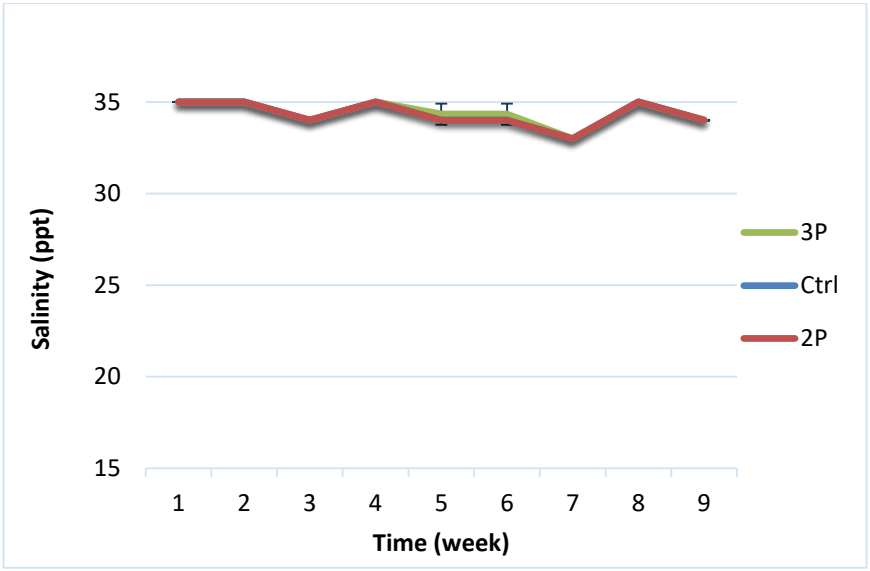


Figure 37. The changes in physicochemical parameters of rearing water: temperature and salinity in all rearing tanks.

5.3.5. Faecal analysis

Total organic content (TOC) in the abalone diet and faecal samples collected during the last seven days of the feeding experiment are presented in Figure 38. In general, there was a significant difference in the TOC of seaweed and faeces, F= 29.63, df 3.8, P<0.05. The TOC in faeces was not significantly different in the abalone receiving either 2P or 3P supplementation, and approximately 13 % lower compared to the control and 20 % lower

than that of *Gracillaria* sp. The TOC in faecal material collected from control group was not significantly different statistically from the diet.

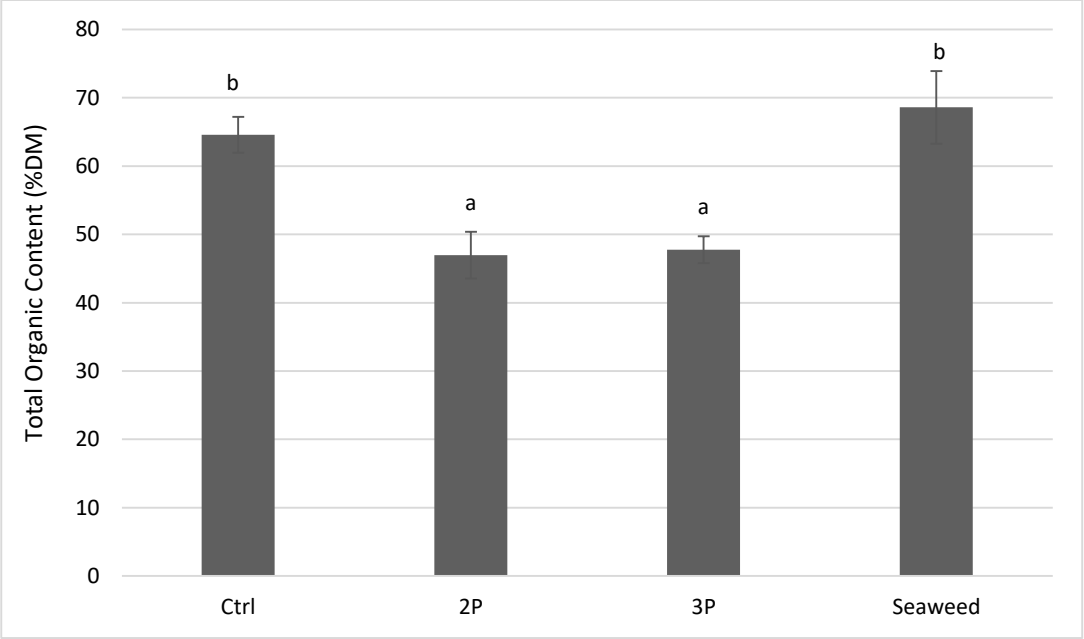


Figure 38. Total organic content in *Gracillaria* sp. and faeces of juvenile abalone receiving either two probiotic candidates (2P), three probiotic candidates (3P) or unsupplemented seaweed as a control (Ctrl). (a:b): treatments with no superscript in common are significantly different, $P<0.05$.

Furthermore, the proximate analysis showed that there was a significant difference in the crude protein (CP) content of seaweed and faeces collected from all treatment groups, $F=41.02$, df, 3,4, $P=0.02$. Overall, the CP values in abalone faeces collected from all treatment groups were significantly lower than the CP observed in the diet, *Gracillaria* sp., Figure 39**.** However, as presented in the figure, there was no significant difference in the CP content detected from faeces among the all treatment groups, and the control.

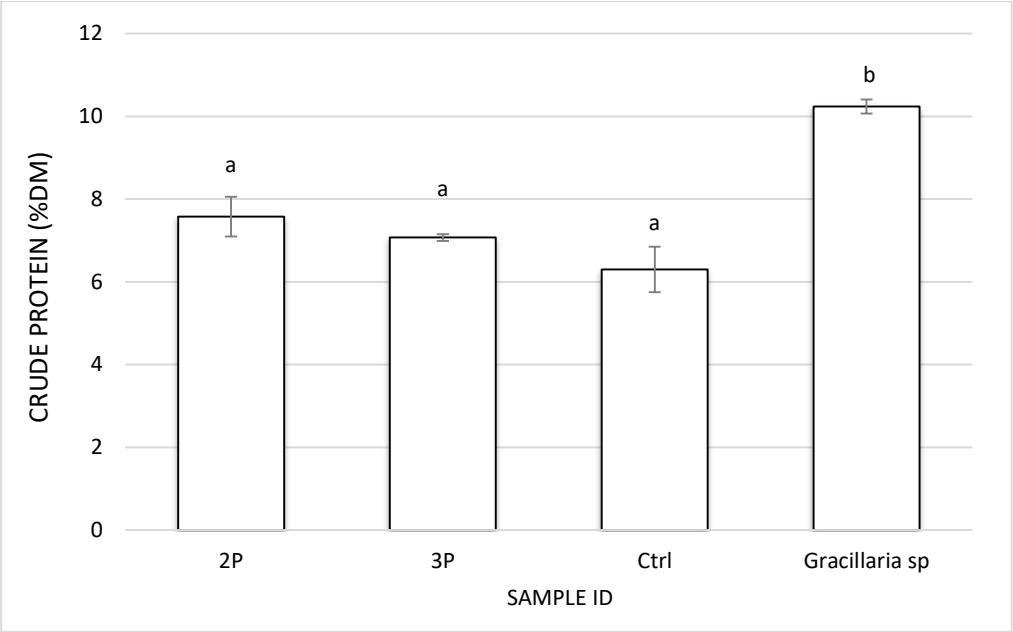


Figure 39. Proximate crude protein of faeces collected from juvenile abalone receiving probiotic. (a:b): Treatments with no superscript in common are significantly different, $P<0.05$.

5.4. Discussion

5.4.1. Growth of abalone

The manipulation of microbiota associated with intestinal tracts using probiotics has been considered as a worthy practice to increase the growth and survival rates of cultivated species (Macey and Coyne, 2005; Wang and Xu, 2006; Ziaei-Nejad et al., 2006; Bagheri et al., 2008; Nimrat et al., 2013; Hamza et al., 2016; Sha et al., 2016). This present study reports the effect of diets containing either two probiotic candidates (two enzyme-producing bacteria: 2P) or three probiotic candidates (2 enzyme-producing bacteria and an acid-producing bacterium; 3P) on the growth and survival rates of tropical abalone, *H. asinina*. The bacteria used in this study originated from the GITs of aquatic species and have been previously confirmed to: (1) produce digestive enzyme or organic acid and antimicrobial compounds, (2) harmless to juvenile abalone, as well as (3) exhibiting a potential capacity to colonize the GITs (stomach and intestine) of abalone. The results showed that the SGR_w

of abalone receiving either 2P or 3P supplemented diet were significantly improved compared to the control. The highest growth rate was obtained from those abalone receiving the 2P-supplemented diet (1.8 % BW d^{-1}), followed by those abalone receiving the 3P-supplemented diet (1.6 % BW d^{-1}), and lastly the control (abalone feed the unsupplemented diet) (1.5 % BW day^{-1}). In terms of shell length, this present study also showed the SGR_L in those abalone receiving either the 2P or 3P supplementation was significantly higher than the control. In addition, total shell gain in the abalone fed on the 2P-suplemented diet was also significantly greater than the abalone receiving the 3P-supplemented diets or the unsupplemented diet of the control.

Total organic content (TOC) in feces of abalone fed with probionts were significantly lower than TOC in the feces of the control, which is in agreement with the higher SGR in the probiotic supplementation. The lower organic content in the feces indicates the higher organic content of feed being absorbed by the abalone. This result suggest that the supplemented probionts could breakdown organic content in the seaweed, or the probionts increased digestibility of the feed (Hamza et al., 2016). Which species of the supplemented probionts contributed more to the feed digestibility was unable to be answered yet, since the alginate content in the feces was not measured. However, based on the CP content in the feces of abalone which was no significant difference between treatment and the control, it is assumed that *E. ludwigii* appeared to be contribute more than *B. amyloliquefaciens* subsp. *plantarum*. However, should be further investigated in future studies.

To the author's knowledge, improved SGR through the use of probiotics has been reported in several abalone species; *H. iris* (Hadi et al., 2014), *H. rufescens* (Silva-Aciares et al., 2011), and *H. midae* (Macey and Coyne, 2005; ten Doeschate and Coyne, 2008). The positive effect of *Exiguobacterium* JHEb1 and *Vibrio* JH1, with a capacity to produce

protease and amylase respectively on the growth of New Zeeland abalone, *H. iris*, was documented by Hadi et al. (2014). Mixture of three probiotic bacteria (*Vibrio* sp. C21-UMA, *Agarivorans albus* F1-UMA and *Vibrio* sp. F15-UMA) administered via seaweed resulted in a significant increase in the growth and survival rates of *H. rufescens* (Silva-Aciares et al., 2011). Furthermore, improved growth and survival rates for *H. midae* was reported due to the incorporation of bacterial and yeast strains with a capacity to degrade protein and starch respectively, by Macey and Coyne (2005), or by the use of *Pseudoalteromonas* sp. (ten Doeschate and Coyne, 2008).

Both the SGR_w and the SGR_L obtained from this present study were higher compared to those previously observed in the same species, Table 19. The SGR_W of *H. asinina* fed with *Gracillaria balinae* was 0.06 % BW day^{-1} (Bautista-Teruel and Millamena, 1999) or 0.7-0.9 % BW day^{-1} when fed pellets with 27 % crude protein content (Bautista-Teruel et al., 2003). In addition, daily growth in terms of weight (DG_w) was also higher compared to that previously documented by Capinpin and Corre (1996).

Table 19. Specific growth rate (SGR), feed conversion ratio (FCR) of juvenile, *H. asinina*.

No	**Iw (g or mm)**	**Diet**	**SGR_W (**%BW/day)	**DG_W (mg/day)**	**FCR**	**Reference**
1	0.52-0.53	*Gracillaria* sp.	1.8 and 1.6	15-17	9 -11	Present study
2	0.68	Pellet	0.75		0.86-1.01	Bautista-Teruel et al (2003)
3	0.48 /14.5	*K. alvarezi*	-	9.2	-	Capinpin and Corre (1996)
4	0.67/15.8	*G. balinae* Pellet	0.06 0.5-0.8		6.98 1.5-2.3	Bautista-Teruel and Millamena (1999)

Iw=initial weight, SGR_W=specific growth rate, FCR=feed conversion ratio, DGw= (final weight-initial weight)/days, DG_L=(final shell length-initial shell length)/ days. "-" is unknown

However, this present study also showed some disagreement with previous studies. For instance, Hadi et al. (2014) compared the effect of two probiotic candidates (protease and amylase-producing *Exiguobacterium* JHEb1, and alginate and amylase-producing *Vibrio* JH1) and three probiotic candidates (the two enzyme producing bacteria and an acid producing *Enterococcus* JHLDc) on the growth of New Zealand abalone, *H. iris*. The study reported that growth of *H. iris* receiving diets supplemented with 3P was superior than the abalone fed on 2P, suggesting the supplementation of organic acid-producing bacterium had significantly improved the growth rates of *H. iris*. To the contrary, this present study showed that the growth rates of abalone receiving additional acid-producing bacterium and two enzyme-producing bacteria were not significantly different to the growth rates of abalone receiving two enzyme-producing bacteria. In addition**,** the FCR value obtained from abalone with 3P supplementation is higher than the FCR calculated for those abalone receiving 2P-supplemented diets.

The difference results beween this study and previous studies might be adressed to several factors. Firstly, the probionts were isolated from temperate regions and applied in a tropical environment. The difference in temperature may affect the growth, colonization, and metabolic activity of probionts (Akerberg et al., 1998; Yan et al., 2007; Altuntas et al., 2010; Miller and McMullen, 2014). It has been reported that *P. acidilactici* grew faster and produce higher amount of bacteriocin-like inhibitory substances in 30 ^{o}C (Altuntas et al., 2010), which is the temperature of rearing water in this present study. Akerberg et al. (1998) also described that *Lactococcus lactis* subsp. *lactis* produced higher fraction of antimicrobial compound (lactic acids) at higher temperature (30 ^{o}C). A study by Yan et al. (2007) showed that temperature highly influence adhesion capacity of *Vibrio* sp. on the foregut and hind gut of fish. Thus, the high rearing temperature in this present study might affect the adhesion capacity of *P. acidilactici* MA160 on the *Gracillaria* sp. or in the gut of abalone. In addition,

one of the probiotic candidates (*P. acidilactici* MA160) produces not only acids but also other antimicrobial compounds which may interfere with the other two enzyme-producing bacteria. Secondly, the difference in animal sources. *P. acidilactici* MA160 was isolated from GIT of Atlantic salmon but was applied abalone. The ability of this strain to attach on the intestinal mucus might be low due to the different source (Rawls et al., 2004). Thirdly, difference in total bacterial load. This present study used total concentrations of ~ 7-8 log CFU g^{-1} diet, which seemed to be lower compared to previous studies, such as 10 log CFU g^{-1} pellet (ten Doeschate and Coyne, 2008) or 12-13 log CFU g^{-1} *Gracillaria* sp. (Silva-Aciares et al., 2011). However, further studies which will count viability of supplemented bacteria in GITs of abalone need to be further investigated.

5.4.2. Mechanism of growth promotion

Although many studies have reported the beneficial effects of probiotics on the growth and survival rates of cultivated species, the exact mechanisms of these actions are not well understood. The improved growth rates of abalone observed in this present study may be related to the production of alginate lyase and protease by *E. ludwigii* and *B. amyloliquefaciens* subsp. *plantarum* respectively, which consequently enhanced the natural digestive enzyme activity of the animal host. Alginate lyase produced by *E. ludwigii* is required to break down alginate content in seaweed into simpler and absorbable glucose (Iwamoto et al., 2001; Kim et al., 2009; Tang et al., 2009). Meanwhile, protease synthesized by *B. amyloliquefaciens* can be used to degrade the protein content in seaweed into absorbable nutrients such as amino acids (Rajasekaran et al., 2014; Shi et al., 2016). Similar results have been reported by Ten Doeschate and Coyne (2008), who observed higher digestive enzyme activity in abalone fed on diets containing the alginate lyase-producing bacterium *Pseudoaltromonas* sp. The same mechanism was also used to explain other studies in which the supplementation of digestive enzyme-producing probionts significantly

elevated the amount of digestive enzymes in the intestine (Iehata et al., 2009), thereby enhancing feed digestion and nutrient absorption, resulting in a better growth rate of cultured animals (Askarian et al., 2011; Sun et al., 2011; Ray et al., 2012; Zhao et al., 2012).

Flye-Sainte-Marie et al. (2007) describe a model of shellfish growth that directs the assimilated nutrients to build up somatic tissue and increase shell growth. However, the amount of nutrients allocated to biomass growth depends on several factors, including the availability of energy sources and the fulfillment of maintenance and other physiological functions (Rico-Villa et al., 2010). In some cases, protein may be burned as an energy source instead of being allocated for the biomass growth (Ogino et al., 1976). In this present study, alginate lyase produced by *E. ludwigii* helped in breaking down alginate into glucose as an energy source. Protein catabolism is more likely to be used for biomass growth, building up somatic tissue (Speth and Spielmann, 1983). Several indicators of this are the lower organic content left in the faeces and the higher growth rate of abalone with probiotic treatment. This present study showed better FCR for the abalone receiving 2P candidates, indicating better feed digestibility and assimilation. However, to clarify this, more studies are necessary.

5.4.3. Probiotic species

Several studies have documented that numerous microflora associated with the GITs of aquatic animals play a significant role in nutrition digestion. Two of three probiotic candidates used in this current study have previously been confirmed as producing digestive enzymes, alginate lyase or protease. In addition, these bacteria have been confirmed to increase the growth and survival rates of cultivated species. For instance, *B. amyloliquefaciens* has been reported to produce a wide range of enzymes, including α-amylase (Palva, 1982), cellulase (Rubio et al., 2009), phytase (Idriss et al., 2002), laccase (Loncar et al., 2013) and fibrinolytic enzyme (Peng et al., 2003). These studies suggested that *B. amyloliquefaciens* could synthesize a wide range of digestive enzymes, which are

very important for enhancing diet digestibility in cultured animals. This was confirmed by Reda and Selim (2015), in which the supplementation of *B. amyloliquefaciens* enhanced protein digestibility, resulting in the improvement of the FCR and growth rates in Nile tilapia, *Oreochromis nilaticus*.

Similarly, *P. acidilactici* MA160 was confirmed to be growth enhancement and to have a protective capacity through a disease challenges and on several aquatic species, including Nile tilapia (Ferguson et al., 2010), shrimp (Castex et al., 2008) and rainbow trout (Merrifield et al., 2011). *P acidilactici* was also reported to inhibit the growth of several pathogens, such as *V. splendidus in vitro* (Villamil et al., 2010), and to outcompete *V. anguillarum* in rainbow trout intestines *in vivo* (Harper et al., 2011).

Liu et al. (2013) reported that *E. ludwigii* has hydrolytic capacity on environmental waste. This species has also been observed to be a potential growth-promoting agent in plants (Shoebitz et al., 2009; de Melo Pereira et al., 2012) as well as having a capacity to produce antimicrobial compounds against fish pathogens (Gosh et al., 2014). As a growth-promoting agent, the bacterium was reported to have antifungal activity and inhibited spore germination (Shoebitz et al., 2009). Another study suggested that exocellular polymer obtained from *E. ludwigii* could be used to hydrolyze a toxin produced by cyanobacteria (Sathya et al., 2015).

There are some recommended studies regarding the result and experimental designs of the 3-probiotic supplementation on the growth and survival rate of abalone. (1). the addition of control, which is non-disinfected *Gracillaria* sp. (two treatments and two controls). There is a possibility that microorganisms attached to the surface of *Gracillaria* sp. might also contribute in the growth or survival rate of abalone. (2) viable count of the administered bacteria should be monitored during the experimental period. (3) measuring external metabolic products of the administered probiotic candidates such alginate lyase,

protease or organic acids) in the GIT of abalone. Measuring these parameters can give better pictures on how probiotic candidates contribute to the growth or survival rate of abalone.

5.5. Conclusion

The first hypothesis was accepted at 95 % of confident interval, in which the growth rate of juvenile abalone, *H. asinina*, fed on *B. amyloliquefaciens* subsp. *plantarum* and *E. ludwigii* (2P)–supplemented *Gracillaria* sp. was significantly higher than abalone receiving unsupplemented *Gracillaria* sp. (control). In addition, the 2P-supplementation showed better value of FCR after a 62-day feeding trial. However, the second hypothesis was rejected, since the growth rates of abalone receiving three probiotic candidates (*B. amyloliquefaciens* subsp. *plantarum*, *E. ludwigii* and *P. acidilactici* MA160) were not significantly different from the growth and survival rates of abalone fed on 2P – supplemented diets. However, the growth of the abalone receiving 3P supplementation was higher than the control. These results suggest that *B. amyloliquefaciens* subsp. *plantarum* and *E. ludwigii* are potential probiotic candidates for abalone.

Chapter 6 : General Summary and Discussion, Limitations, and Future Recommended Studies

6.1. General summary

Viability of probionts in GITs of aquatic species is still considered to be major challenge when probionts are applied in aquatic animals (Balcázar et al., 2007; Iehata et al., 2010; Silva-Aciares et al., 2011). To deal with this issue, many studies recommended that probionts for aquaculture species should be isolated from GITs of aquatic species (Spanggaard et al., 2001; Fjellheim et al., 2010). Therefore, this study aimed at isolation and screening of endogenous bacteria associated with GITs of aquatic animals as probiotic candidates.

6.1.1. Isolation of endogenous bacteria

This study was started by isolating endogenous bacteria from the GITs of 155 teleosts and molluscs collected around Tasmanian water, Australia (Chapter 2) using two culture-dependent approaches, a direct spreading method and an enrichment method. Twenty isolates were obtained using direct spreading method and 181 isolates were obtained using the enrichment culture method. A total of 230 bacteria were obtained from both approaches: 24 enzyme-producing bacteria and 206 LAB. Twenty-five LAB were obtained with the direct spreading method, and 205 were obtained using enrichment method. Based on their 16S rDNA sequences, the 230 isolates were classified to 15 genera: *Achromobacter, Bacillus, Carnobaterium, Enterococcus, Enterobacter, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Pseudomonas, Shewanella, Shigella, Serratia* and *Stenotrophomonas,* and *Weisella*. These endogenous bacteria were then screened for production of beneficial compounds, either digestive enzymes or antimicrobial compounds.

6.1.2. Enzyme-producing bacteria

The screening assay indicated 24 isolates showed capacity to produce one of three digestive enzymes; protease (11 isolates), cellulase (7 isolates) or alginate lyase (6 isolates).

The enzyme activity of these 24 isolates was then investigated by measuring their capacity to degrade casein, CMC, and sodium alginate for protease, cellulase and alginate lyase respectively. Of these, three isolates were selected for further characterization due to displaying the highest enzyme activity in either protease, cellulase or alginate lyase (Chapter 3). The selected endogenous bacteria were identified as *B. amyloliquefaciens* subsp. *plantarum*, *S. maltophilia* and *Enterobacter ludwigii* for protease, cellulase and alginate lyase production. The results showed that these bacteria displayed good tolerance to the high salinity (32 ppt) of abalone rearing water, high stability in manufactured pellet stored at 4^{o}C, and good tolerance of the low pH, gastric enzymes (trypsin and pepsin) and bile salts in the simulated GITs of abalone. In addition, these enzyme-producing bacteria showed their susceptibility to chloramphenicol, as well as being harmless to juvenile abalone as determined by an *in vivo* study.

6.1.3. Antimicrobial compound-producing bacteria

Twenty-two LAB had inhibitory activity against at least one of eight bacterial pathogens (Chapter 4). Two LAB were obtained using the microtiter plate assay and the other 20 LAB have been observed using well diffusion assay. These bacteria were identified as 14 species, and all belonged to members of LAB. Of these, two isolates (*P. acidilactici* MA160 and *P. pentosaceus* MA169) were chosen to be further studied, due to displaying broad spectra of antimicrobial activity, especially to five vibrios commonly infecting marine aquaculture species including abalone.

The antagonistic activity appeared due to organic acids and BLIS. The presence of organic acids was indicated by the lower pH of the broth culture after being inoculated with these LAB. In addition, the antimicrobial activity which remained active when pH of CFS was neutralized, indicated the presence of BLIS. The capacity of these pediococci to produce bacteriocin was supported by PCR results, in which a gene which encodes one of the most

common bacteriocin produced by genus *Pedicococcus,* Pediocin-PA1 was detected from both pediococci. The amplified gene size was 500 and 1000 bp for *P. acidilactici* MA160 and *P. pentosaceus* MA169, respectively.

Furthermore, i*n vitro* study indicated that the two pediococci showed good viability in the simulated gastric juices (SSJ and SIJ), indicated by the high survival rate after being incubated in the simulated stomach and intestinal juices. These pediococci also exhibited good adhesion capacity as well as being able to grow in intestinal mucus, which may indicate that they have potential capacity to outcompete pathogens not only by antimicrobial-compound production, but also by competitive exclusion.

6.1.4. Confirmation of *in vitro* results by *in vivo* studies

Three bacteria namely *B. amyloliquefaciens* subsp. *plantarum, E. ludwigii* and *P. acidilactici* MA160 were selected for an *in vivo* study. *B. amyloliquefaciens* subsp. *plantarum* and *E. ludwigii* were selected due to their ability to produce protease and alginate lyase, respectively. Previous studies suggest that the growth of abalone can be enhanced by supplementation of protease-producing bacteria and alginate lyase-producing bacteria (Erasmus et al., 1997; Macey and Coyne, 2006; Hadi et al., 2014; Huddy and Coyne, 2015). *P. acidilactici* MA160 was selected due to its ability to produce organic acids, antimicrobial compounds, and also the only strain which have been authorized by EU commission for aquaculture industries. In addition, based on a study by Goosen et al. (2011), organic acids in feeds can be a potential growth promoters in the South African abalone.

Three bacteria have been previously confirmed that they would have high viability in the GIT of abalone indicated by their high tolerance in the SSJ and SIJ. In addition, these bacteria were harmless, and not carrying chloramphenicol-resistance gene. In addition, *P. acidilactici* MA160 exhibited good adhesion capacity as well as being able to grow in intestinal mucus, which may indicate that they have potential capacity to outcompete

pathogens not only by antimicrobial-compound production, but also by a competitive exclusion. Furthermore, the strain also is one of bacterial strains which has been approved by European commission for probiotic in humans.

These bacteria were added to abalone diet (*Gracillaria* sp.) at concentrations of 7-8 log units (CFU g^{-1}), and administered to juvenile abalone in mixtures of two (*B. amyloliquefaciens* subsp. *plantarum* and *E. ludwigii*; 2P) or three (*B. amyloliquefaciens* subsp. *plantarum, E. ludwigii* and *P. acidilactici* MA160: 3P) for 62 days. The results showed that abalone receiving either 2P or 3P had significantly higher growth rates (both weight and shell length) compared to the control, as presented in Table 18 and 19. Among the treatments, abalone fed on 2P-supplemented diet appeared to grow more than abalone receiving 3P-supplemented diet. FCR calculated from those abalone fed 2P-supplemented diet were also significantly lower than those abalone receiving either 3P or unsupplemented diets (Chapter 5). However, the present study showed no significant difference in survival rate between the abalone receiving probiotic-candidate treatments and those abalone in the control. The survival rate of abalone was recorded at 78% (2P supplementation), 80% (3P supplementation), and 72% (Control).

Overall, the best growth was obtained from those abalone fed on *Gracillaria* sp. supplemented with a mixture of *B. amyloliquefaciens* subsp. *plantarum* and *E. ludwigii*. However, none of the bacterial supplementations significantly improve in the survival of juvenile abalone.

6.2. General discussion

6.2.1. Isolation methods: Direct spreading method vs Enrichment culture method.

This study used two different approaches (direct spreading method vs enrichment culture method) to isolate intestinal bacteria which have a potential capacity to produce either digestive enzymes or antimicrobial compounds (Chapter 2). Enzyme-producing bacteria were obtained only using the enrichment culture method. While, no enzyme-producing bacteria could be isolated using the direct spreading method, which may suggest that the number of enzyme-producing bacteria in the GITs of samples are very low (Chen et al., 2005). In contrast, previous studies reported the high number of enzyme-producing bacteria associated with the GITs of aquatic animals such as carp, tilapia and catla, therefore being able to be detected using the direct spreading method (Bairagi et al., 2002; Kar and Ghosh, 2008; Mondal et al., 2008; Ray et al., 2010; Gupta et al., 2012; Rajasekaran et al., 2014). The differences in the result of this study and previous studies might be due to: difference of size and species of animal samples, diets, and environmental conditions from which samples was taken. Such differences have been also reported from previous studies. Cellulolytic bacteria, for instance, were not detected from the GITs of *C. punctatus* (Bairagi et al., 2002). Meanwhile, Kar and Ghosh (2008) isolated a high number of cellulolytic bacteria from the GITs of the same species. The difference in intestinal microbiota might be addressed to diets of animal. Bairagi et al. (2002) reported a positive correlation between feed habits and the number and diversity of enzyme-producing intestinal bacteria. Furthermore, the other possibility (2) is the difference of culture media which was used. Different media might support the growth of different bacterial strains (Carvalho et al., 2003).

Meanwhile, LAB could be isolated using both culture-dependent approaches, (Chapter 2). The average number of LAB isolated from Atlantic salmon was 1.10×10^2 CFU

g^{-1} intestine, and 1.65 x 10^2 CFU g^{-1} intestine from GITs of wild seabream. This number was much lower compared to some previous studies. Askarian et al. (2009) reported minimum value of LAB in GITs of 200-300 g belunga was 2.3 x 10^4 CFU g^{-1} intestine. In addition, Bucio et al. (2006) reported that the number of LAB isolated from GITs of carp was 4.6 x 10^4 CFU g^{-1} intestine using MRS 42 medium.

Overall both approaches have advantages and this advantages in isolating bacteria from GITs aquatic species. The direct spreading method can give an approximate number of LAB in the sample, but this method also cannot be used in sample with low number of bacterial target. While the enrichment culture method can be used to isolate low number of bacterial target in sample (Chen et al., 2005), but their number are not known. Thus, combining both approach to isolate bacterial target are highly recommended.

6.2.2. Screening of antimicrobial compounds

This study used either microtiter plate assay or well-diffusion assay to screen antagonistic LAB. The result showed that only ten percent of total LAB isolates were obtained (chapter 4). In contrast, Klaenhammer (1988) reviewed that 99% of LAB have at least one bacteriocin-encoding gene. However, Blanchard et al. (2016) also suggested that such bacteriocin-encoding genes are possibly not expressed because of the large amount of nutrient and energy required to synthesize the bacteriocin. In addition, other studies explained that many factors such as temperature and bacterial inducers can affect the expression of bacteriocin-encoding genes (Miller and McMullen, 2014; Chanos and Mygind, 2016; Perin et al., 2016). As the present study used a phenotypic test with no selective pressure during growth, it may block the expression to conserve energy.

Balouiri et al. (2016) revised that there are many different methods which could be used to evaluating antimicrobial activity such as microtiter plate assay, well-diffusion assay,

disk-diffusion assay and broth or agar dilution method. Each method has advantages and disadvantages. Therefore, in order to obtain more comprehensive result, it is suggested to used more than one screening method in parallel.

6.2.3. *In vitro* screening of probiotic viability

Several studies concluded that probionts should be alive in order to optimally elicit desired beneficial products or activities (e.g., Panigrahi et al., 2005). Therefore, maintaining probiont viability is another important challenge when probionts are administered to aquatic animals (D'Orazio et al., 2015). The application of probionts in aquatic animals are generally delivered through diets (Carnevali et al., 2004) or directly added to the rearing water (Verschuere et al., 2000a). For some strains with less tolerance to different environmental conditions, more advanced techniques such as microencapsulation are introduced to maintain their viability until reaching target sites (Chen et al., 2012; Rosas-Ledesma et al., 2012; D'Orazio et al., 2015; Coghetto et al., 2016). However, this technique leads to additional cost in a probiotic approach. To avoid laborious work and cost, probionts which have better tolerance to various environmental conditions to which they are exposed during delivery processes are required. The result of this present study showed that the 2 enzyme-producing bacteria (*S. maltophilia* and *E. ludwigii*) maintained their high viability when they were mixed with commercial pellets and stored at 4 ^{o}C for 7 days. These bacteria also had high resistance to salinity of 32 ppt, which suggests these bacteria can be administered either via the diet or by directly adding to the rearing water. However, bacterial losses from feed due to leaching in water still needs be further studied, to predict the density reaching the intestinal tract.

Meanwhile, *B. amyloliquefaciens* subsp. *plantarum* appeared to have low viability in feed, and SIJ (chapter 3). However, as *Bacillus* are most likely be used as spore, supplementation of this strain can be done in the form of spores. The use of spores has been

reported in several studies including the use of spores of *B. pumilus* (Prieto et al., 2014) and *B. amyloliquefaciens* for striped catfish, *Pangasianodon hypophthalamus* (Truong Thy et al., 2017). The mechanism for producing spore preparations from this bacterial strain and the effect of spore supplementation will be further studied.

Additionally, probiotic candidates should be able to survive passage through stomach conditions before reaching the intestinal tract where digestion and absorption of feed occur. Several studies suggested that low pH in stomach, and the presence of bile salt and gastric enzymes such as pepsin and trypsin in the intestinal tracts, are toxic to some bacteria (Giannella et al., 1972; Borriello et al., 1985; Nikoskelainen et al., 2001b; Fjellheim et al., 2010; Geraylou et al., 2014; Saito et al., 2014). Hence, these parameters were used to make a simulation of the GIT (stomach and intestine) to evaluate viability of probiotic candidates by *in vitro* assay. This study showed that the three enzyme-producing bacteria were resistant to the simulated stomach juice (pH of 5), which is stomach pH of abalone (Harris et al., 1998), and also showed high tolerance to the simulated intestinal juice containing bile salts as well as pepsin and trypsin with a pH value of 6.65, which is intestinal pH of abalone (Harris et al., 1998). The high tolerance of the tested bacteria to bile salt and gastric enzymes might be due to their capacity to develop a protective mechanisms such as a protective coating of exopolysaccharides (Roberts and Powell, 2005) or their ability to produce bile salt hydrolase, an enzyme which could reduce the toxicity of bile salt (Geraylou et al., 2014). These results indicate that the three bacteria are highly likely able to reach and live in the intestinal tract of abalone.

Furthermore, this study displayed that the two pediococci (antimicrobial compound-producing bacteria) were resistant to low pH (4), lower than pH of abalone stomach, 5 (Harris et al., 1998), and pH of 7.4 in SIJ which was higher that pH of abalone intestine, 6.65 (Harris et al., 1998). In addition, the pediococci were able to adhere and grow in the intestinal

mucus. Previous studies concluded that colonization potential of probiotic candidates can be evaluated by measuring the capacity of the probiotic candidates to adhere and grow in intestinal mucus (Olsson et al., 1992; Geraylou et al., 2014). Thus, this study may indicate that the pediococci have potential capacity to colonize intestinal tracts.

Several authors describes that probionts can be antagonistic to pathogens by either inhibiting or killing bacterial pathogens through releasing antimicrobial compounds (Gildberg and Mikkelsen, 1998; Nikoskelainen et al., 2001a; Sica et al., 2012; Touraki et al., 2012; Maeda et al., 2014), or by competitive exclusion, indicated by good adhesion activity on the target sites (Verschuere et al., 2000b; Panigrahi et al., 2005; Swain et al., 2009). In this present study, the pediococci was observed to have good adhesion capacity to intestinal mucus, which may suggest another antagonistic mechanism to protect cultivated animals from pathogenic invasions, besides producing antimicrobial compounds. However, this possibility needs to be further studied through disease challenge by *in vivo* experiment.

6.2.4. The application of probionts in abalone

The use of probionts to enhance the growth and disease resistance of abalone have been documented in several studies including *H. midae* (ten Doeschate and Coyne, 2008), *H. gigantea* (Iehata et al., 2009), and *H. rufescens* (Silva-Aciares et al., 2011), *H. discuss hannai* (Jiang et al., 2013a), and *H. iris* (Hadi et al., 2014). However, to author‘s knowledge, no probiotic study has been done in *H. asinina*.

This present study has confirmed that supplementation of 2 enzyme-producing bacteria (*B. amyloliquefaciens* subsp. *plantarum* and *E. ludwigii*) isolated from the GIT of abalone significantly improved the growth, FCR, and total weight gain of juvenile abalone, *H. asinina*. The growth of abalone receiving the 2 probiotic candidates was higher than the control, and two times higher, compared to the growth of *H. asinina* previously reported without probiotic supplementation. Weight gain of *H. asinina* after being fed with the 2

probiotic candidates (2P) was recorded at 17.3 mg day^{-1} (1.07g in 62 days for abalone receiving 2P-supplemented diet). Previously, it was reported that weight gain of *H. asinina* with the same initial size (~0.5 g) fed on *Gracillaria heterolada* without probiotic supplementation was only 9.2 mg day^{-1} (Capinpin and Corre, 1996). In addition, the SGR calculated in this present study was 1.75 %BW day^{-1} which is two times higher compared to SGR of *H. asinina* fed with formulated diet without probiotic supplementation, 0.75 %BW day^{1} (Bautista-Teruel et al., 2003) and 0.5-0.8%BW/day when receiving *gracillariopsis bailinae* (Bautista-Teruel and Millamena, 1999). These results indicate that the growth of *H. asinina* can be improved by supplementing with endogenous probionts (*B. amyloliquefaciens* subsp.*plantarum* and *E. ludwigii*).

The higher growth of abalone receiving 2P supplementations can be explained by the lower amount of total organic content (TOC) in the faeces of abalone supplemented with either 2P or 3P, compared to faeces of abalone fed without probionts. This result indicates that the probiont supplementation has enhanced the feed digestion of the abalone by supplying additional digestive enzymes to the intestinal tract of abalone, which later helps digest the macroalgae. The same mechanisms have been previously described from other aquatic animals, in which the inclusion of enzyme-producing probionts increased growth of their hosts by excreting additional digestive enzymes, enhanced digestion and absorption of feed, and in turn contributed to the improved FCR and SGR (Suzer et al., 2008; Merrifield et al., 2010a; Nimrat et al., 2012; Ridha and Azad, 2012; Lara-Flores and Olvera-Novoa, 2013; Hadi et al., 2014; Hamza et al., 2016). In addition, alginate of seaweed consists of polymannuronate (polyM) block and polyguluronate (polyG) block, and it has been previously described that abalone are unable to digest the polyG (Sawabe et al., 1995). However, several bacterial strains associated with GIT of abalone have been observed to have capacity to digest polyG (Sawabe et al., 1995; Erasmus et al., 1997). This study did not

investigate the ability of these alginate lyase-producing bacterium to degrade polyG in macroalgae. Additional studies of polysaccharide degradation probiotic candidates are required to elucidate the precise mechanism by which they aid digestion in *H. asinina*.

The other possible mechanism is probionts lower intestinal pH, resulting in improving nutrient solubility and nutrient absorption (Lee et al., 1999; Barroso et al., 2016). In addition, probionts have been reported to modify the structure of intestinal epithelial cells including increasing perimeter ratio, and enlarge diameter of microvilli which lead to the increasing of enterocyte absorptive area in Tilapia (Adeoye et al., 2016; Barroso et al., 2016). The same mechanism was proposed by Hadi et al. (2014) to explain their result, where the growth and survival rate of New Zealand abalone, *H. iris* have been improved by the inclusion of acid-producing *Enterococcus* sp. However, in the present work, supplementation of acid-producing *P. acidilactici* MA160, resulted in no improvement in growth compared to supplementation with nonacid-producing bacteria. The survival of abalone receiving *P. acidilactici* MA160 supplementation was also not significantly different from the control or with the 2P-supplemented diets. Several possible reasons are: (1) abalone in this study were not subjected to a disease challenge (e.g. vibrios). (2) *P. acidilactici* MA160 was isolated from a different species, Atlantic salmon; (3) environmental factors especially temperature and salinity from which the bacterial strain was isolated and screened differed from tropical conditions to which it was tested (*in vivo* study). This could have implications for its growth characteristics and production of antibacterial compounds. and (4) some abalone mortality was observed due to walking activity (abalone leaving water during dark periods), a phenomenon recorded in studies of *H. laevigata* (Bansemer et al., 2015) and *H. midae* (Britz et al., 1997). Thus, improved shelter structures that prevent escapes are highly recommended for further studies. An important point that can be taken from this study is that *P. acidilactici* MA160 was harmless towards the juvenile

abalone. Since this bacterial strain has been confirmed to not only produce organic acids, but also other antimicrobial compounds active against vibrios, a pathogen-challenge experiment should be undertaken to determine whether this probiont can protect against vibrio-related disease.

6.2.5. Probionts as potential approach to enhance abalone performances

The successful application of probiotic supplementation has been reported in many commercial aquatic species including several species of abalone: *H. iris* (Hadi et al., 2014), *H. gigantea* (Iehata et al., 2010), *H. midae* (Macey and Coyne, 2005; ten Doeschate and Coyne, 2008), *H. discuss hannai* Ino (Jiang et al., 2013a) and *H. rufescens* (Silva-Aciares et al., 2011). The studies indicated that the probiotic inclusions significantly improved the growth or disease resistance of abalone. However, compared to other cultivated aquatic species, the extent to which this method has been used so far in abalone still considered to be limited. Based on the present and previous studies, probionts can increase growth rate and thus have potential to shorten grow-out times, leading to lower production costs and improve economic sustainability of abalone aquaculture. In addition, probionts are potential alternative to drug and antibiotics for controlling of infection. In general, development of probionts for abalone is of great importance for the future development of abalone aquaculture and responsible management of environmental impacts of abalone aquaculture.

6.3. The limitations of this study

6.3.1. Medium of isolation

The are several media with different components and protocols developed by several authors to isolate enzyme-producing bacteria. For instance, media for isolating extracellular amylase, protease, cellulase, lipase or alginate lyase have been described in previous studies by several authors (Hoshino et al., 1997; Bairagi et al., 2002; Tang et al., 2009; Zhou et al.,

2009; Sirisha et al., 2010; Alnahdi, 2012; Hadi et al., 2014). Different media and protocols might support the growth of different bacteria. However, this present study only used one source of medium and protocol. This might be the reason why less diverse of enzyme-producing bacteria was obtained in this present study. Therefore, combination of several protocols is highly recommended in future study, to obtain more number and diverse results.

LAB targeted for antimicrobial compound production are generally known to be auxotrophic bacteria, having limited capacity to synthesize nutrients such as amino acids from inorganic sources (LeBlanc et al., 2011). Thus, the bacterial strains that can be recovered is highly dependent on the growth medium used. This present study used two commercial media: De Man Rogosa Sharp (MRS, Oxoid) and M17 (Oxoid). Previously, MRS medium has been described to support the growth of only 4 genera: *Lactobacillus, Streptococcus, Pediococcus* and *Leuconostoc* (De Man et al., 1960), and M17 medium for *Streptococcus thermophilus*, *Lactobacillus delbrueckii*, and *Lactococcus lactis* (Terzaghi and Sandine, 1975). Therefore, using only these two-commercial media may lead to an underestimate of the number and diversity of LAB associated with the GITs of aquatic species. The use of wider range of media, such as BM medium (Yanagida et al., 2008), glucose yeast extract peptone (GYP) medium (Maeda et al., 2014), Enterococcus medium (Litsky et al., 1953), and Raka Ray (Abassah-Oppong et al., 2007) may have increased the diversity and number of isolates.

In addition, the use of molecular approaches might detect the presence of more LAB than were detected using culture-dependent methods. A study by González-Arenzana et al. (2013) demonstrated that polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) detected 9 out of 11 LAB, while culture-dependent method using a modified MRS medium could detect only 5 out of 11 LAB. Based on these studies, it is therefore recommended to use a polyphasic approach which combines molecular (e.g., PCR) and

culture-dependent methods with more varieties of culture media, to give a more comprehensive profile especially to LAB associated with the GIT of aquatic animals.

6.3.2. Screening methods

Screening of antagonistic bacteria was performed using either the microtiter plate assay or well diffusion assay. Both method used only extracellular products in terms of CFS or CFSn of tested bacteria. Meanwhile, there are many other antagonistic mechanisms such as immunomodulation and competition for adhesion site and nutrients which should be tested using live cells (Kesarcodi-Watson et al., 2008). However, all these mechanisms cannot be investigated in this present study. Furthermore, the non-antagonistic bacteria (result from this present study) does not necessary means that they do not have capacity to produce antimicrobials. The non-antagonistic bacteria might carry antimicrobial-encoding genes but the expression of extracellular antimicrobial compounds (e.g., bacteriocin) might be inhibited by the culture condition or nutrient which were available in the culture medium. As consequence, screening of antagonistic bacteria using well diffusion or microtiter plate assay was frequently reported to underestimate the number of antimicrobial-producing bacteria (Miller and McMullen, 2014; Chanos and Mygind, 2016; Perin et al., 2016). Therefore, more screening methods including immunomodulation, competition for adhesion and nutrient, and molecular technique are highly recommended for future studies to get more comprehensive result.

Furthermore, the screening of digestive enzyme-producing bacteria was based on their capacity to utilize substrates: casein and gelatin (protease), alginate lyase (sodium alginate) and carboxymethyl cellulose (cellulase). The casein, gelatin and CMC used in this screening assay might be different to the substrates used in the natural diet and the manufactured diet of aquaculture animals. For instance, it was described that a protein substrate from different sources may have a different structure (Ochoa-Solano and Olmos-

Soto, 2006). However, this current study did not investigate the capacity of the 24 enzyme-producing bacteria to digest fish meal or soybean meal by protease activity nor for cellulase activity on fresh *Gracillaria* sp. Selected bacterial strains based on these substrates (*Gracillaria* sp., fish meal or soybean meal) might give more applicable results for further *in vivo* studies, especially for abalone.

6.3.3. Characterization of digestive enzymes and antimicrobial substances

This present study identified 24 isolates which could produce one of these enzymes; cellulase, protease and alginate lyase. The screening assay was performed all by *in vitro* assay. Verschuere et al. (2000a) reviewed that results of *in vitro* studies should be confirmed by *in vivo* assay to get more valid result. However, this study did not investigate the 24 enzyme-producing bacteria by *in vivo*. For instance, whether the bacterial strains could produce digestive enzyme in environmental condition of GITs or in what conditions do the bacterial strains produce the digestive enzymes optimally. In addition, the specific mechanisms of protease, cellulase or alginate lyase which were produced by the isolated strains were not studied. For instance, there two types of alginate lyase, poly(α-L-guluronate)lyase which act on PolyG, and poly(β-D-mannuronate)lyase which act specifically on PolyM (Iwamoto et al., 2001). It has been described that abalone need poly(α-L-guluronate)lyase, because alginate lyase produced endogenously could only digest polyM (Sawabe et al., 1995). Thus, further characterization of digestive enzymes should be investigated in future studies.

Furthermore, the antimicrobial substances produced by the 22 endogenous bacteria and digestive enzymes produced by the 24 endogenous bacteria are still poorly characterized. In terms of antimicrobial substances, a number of studies have documented that LAB produce several antimicrobial substances such as organic acids (Goncalves et al., 1997;

Vazquez et al., 2005), bacteriocins (Verschuere et al., 2000a; Lin et al., 2013), and hydrogen peroxide (Verschuere et al., 2000a). This current study confirmed the presence of organic acids, indicated by a lower pH in the cell-free supernatant of LAB culture (De Vuyst and Vandamme, 1994; Presser et al., 1997). The antagonistic activity of hydrogen peroxide was excluded since the CFS or CFS was obtained from anaerobic culture condition. The other possible antimicrobial substances are bacteriocin-like inhibitory substances (BLIS), indicated by the presence of antimicrobial activity after the CFS of each LAB was neutralized (pH ~6.5-6.8). However, quantification of organic acids by these 22 LAB was not studied. In addition, Pediocin PA-1 encoding gene detected from two pediococci, *P. acidilactici* MA160 and *P. pentosaceus* MA169 should be further confirmed by sequencing the amplified genes. Furthermore, the expression of the bacteriocin-encoding gene should be tested by treating with proteinase K.

6.3.4. Viability of the supplemented probionts

The viability of three supplemented bacteria (*B. amyloliquefaciens* subsp. *plantarum*, E. *ludwigii* and *P. acidilactici* MA160) in the GIT of juvenile abalone was not monitored. Even though these three probiotic candidates were previously confirmed to have a high tolerance to the low pH in the stomach as well as to the bile contents in intestinal tract, the *in vitro* assay was done in a sterile environment (chapter 3 and 4). On the other hand, a diverse group of endogenous bacteria inhabit the abalone GIT and may interfere in terms of nutrient and space competition. One of the supplemented bacteria (*P. acidilactici* MA160) has also been confirmed to release antimicrobial substances against a broad range of bacterial pathogens, such as *Vibrio*, *Yersinia,* and *Listeria*. This current study did not evaluate whether *P. acidilactici* MA160 was antagonistic to the other two supplemented bacteria. All these factors may determine the contribution of supplemented probionts. While it was suggested

that interactions among 2 or more probiotic strains should be taken into account when they were used in mixture (Makridis et al., 2000).

This present study did not quantify the amount of digestive enzymes, especially protease and alginate lyase, in the intestinal tracts of juvenile abalone in both treatments and the control group. The comparison of these enzyme amounts could be used to evaluate the activity and the capacity of the supplemented probionts to aid feed digestion (Wang, 2007; Kar and Ghosh, 2008; Askarian et al., 2011).

6.4. Future recommended studies

6.4.1. Screening for antimicrobial compounds using combination of several screening methods

This present study used only one screening method to screen antimicrobial production, either the microtiter plate assay or the well diffusion assay. Screening of antimicrobial production using combination of several techniques such as well diffusion assay in parallel with microtiter plate assay, disk-diffusion assay, and broth or agar dilution method might give more number of antimicrobial-producing strains. It is also recommended that the screening assay uses not only extracellular products but also live cells (Hamid et al., 2012) and cellular component of tested bacteria (Giri et al., 2011). In addition, the screening of antimicrobial production should be performed to the 24-digestive enzyme-producing bacteria. One of the isolates, *B. amyloliquefaciens,* for instance has been reported to not only synthesize digestive enzymes (amylase, protease and cellulase), but also could produce antimicrobial compounds (Wang et al., 2002; Yu et al., 2002; Lisboa et al., 2006; Xu et al., 2014). Furthermore, testing if any of antagonistic strains inhibits other strain should be studied before deciding on combinations to test *in vivo*.

6.4.2. Screening of LAB for production of other beneficial substances

LAB produce a wide range of beneficial metabolic products, not only antimicrobial substances and digestive enzymes, but also other beneficial substances, including vitamins (Hugenschmidt et al., 2010; LeBlanc et al., 2011; LeBlanc et al., 2013), amino acids and short-chain fatty acids (SCFAs) (Iehata et al., 2009), siderophores (Verschuere et al., 2000a), growth-promoting substances, anti-carcinogens (Hirayama and Rafter, 1999), and immune-stimulating substances (Rowley and Powell, 2007; Maeda et al., 2014). In the present study, LAB were only screened for production of antimicrobial compounds and digestive enzymes; screening for other beneficial extracellular products (e.g., vitamins, short-chain fatty acids and carcinogen binders) should be further investigated. These compounds can beneficially contribute to an animal host through either enhanced disease resistance or increase in the growth of cultivated animals. A study by Iehata et al. (2010) reported that SCFAs contributed to meet energy requirements in abalone. In addition, *Pseudomonas* spp. producing siderophores can outcompete bacterial pathogens for iron requirements (O'Sullivan and O'Gara, 1992; Verschuere et al., 2000a).

6.4.3. Supplementation of several bacterial combinations

Gracillaria sp. and other red algae are the most preferred natural diet of abalone. However, the digestibility of the red algae is generally low, indicated by relatively high FCR of 7 to 9 (Bautista-Teruel and Millamena, 1999). It is generally accepted that plant-based diets such as *Gracillaria* sp. composed of 2.4-5.3 % cellulose (Siddhanta et al., 2011) is difficult to digest. The cellulose content has been associated with several adverse effects on digestive processes, including increasing the viscosity of intestinal juice that caused a reduction in the access of digestive enzymes to other feed materials (Bromley and Adkins, 1984) and lowering the feed intake, resulting in slowing the growth of cultured animals (Francis et al., 2001). In addition, Sawabe (1995) reported that alginate in macroalgae

consists of polyG and polyM block, and endogenous alginate lyase produced by abalone only able to degrade polyM block, but polyG block. However, to degrade and harvest energy sources from PolyG block, abalone need external enzyme supply from bacteria associated with the GIT of abalone.

Few studies have concluded that the cellulose content in natural abalone diets, such as *Gracillaria verucosa*, and *Palmaria palmata*, can be degraded by adding cellulase enzymes (Fleurence et al., 1995). Additionally, the polyG block in macroalgal polysaccharides able to be degraded by some bacteria (Sawabe et al., 1995). Acknowledging this issue, this current study has isolated seven bacterial strains which have the capacity to degrade cellulose, thereby disrupting the cell walls of abalone feed such as *Gracillaria* sp. Thus, a study which combines cellulose-degrading bacteria, alginate-degrading bacteria and protein-degrading bacteria might give better growth rates than what was achieved by supplementation of only alginate lyase and protease-producing bacteria. The inclusion of these bacteria could lead to the development of low-cost formulated diets, such as plant-based formulated diets. However, a proper combination of these three enzyme-producing bacteria should be firstly studied.

6.4.4. *In vivo* studies

This present study detected the presence of 46 bacteria exhibiting the capacity to produce either digestive enzymes or antimicrobial compounds from GITs of aquatic animals, consisting of: 24 enzyme-producing bacteria and 22 antimicrobial compound-producing bacteria. Of these, five bacteria were studied for their resistance to the low pH and bile salt content in the GIT of abalone, and only three of them were tested by an *in vivo* study using juvenile abalone (two enzyme-and one antimicrobial-producing bacteria). These bacteria were selected only based on their performances on *in vitro* studies. However, several studies reported that neither positive nor negative *in vitro* results may predict the actual effect of *in*

vivo results (Verschuere et al., 2000a; Balcazar et al., 2006). Many environmental factors such as the availability of nutrients, temperature, pH, and growth phase may interfere with the results of the *in vitro* studies (Biswas et al., 1991; Devi and Halami, 2011). Therefore, more *in vivo* studies using the 46 endogenous bacteria (single or combined bacteria) need to be further investigated on the growth and survival of abalone and other aquatic animals.

This present study was unable to isolate bacteria that produced lipase and amylase. These enzymes are also important enzymes which particularly required to digest lipid and carbohydrate into simpler and absorbable compounds by the animal hosts (Ray et al., 2010; Singh et al., 2010). Amylase are required to break down carbohydrate and lipase to digest lipid to be source of energy, essential fatty acids and lipid-soluble vitamins (Bairagi et al., 2002). Therefore, besides the *B. amyloliquefaciens* subsp. *plantarum* and *E. ludwigii*, the addition of amylase or lipase-producing bacteria may give higher feed digestibility and better growth rates in abalone.

In addition, a challenge experiment to study the protective capacity of probiotic supplementation against specific bacterial pathogens, especially to those which infect abalone, is needed. This sort of trial will determine whether antimicrobial compound-producing bacteria isolated in this present study have useful protective capacity against pathogens, and whether they will interfere with enzyme-producing bacteria.

6.5. Concluding statement

This present study confirms the presence of diverse bacteria associated with the GITs teleosts and molluscs that appear to contribute to feed digestion. these beneficial bacteria appear not to be dominant in the GIT of abalone or other aquatic animals, indicated by the fact that these bacteria can only be isolated using an enrichment culture method. Increasing cell numbers of digestive enzyme-producing bacteria in the GIT of abalone through feed

supplementation for 62-day feeding experiment significantly increased feed digestibility and the growth of juvenile abalone.

Acknowledging the presence of either enzyme or antimicrobial compound-producing bacteria (contributing to the growth or disease resistance) in the GIT of teleosts and molluscs including abalone, another possibility which can be used to increase cell numbers of the beneficial bacteria in the GIT of abalone is by supplying sufficient amounts of the nutrients required to optimize the growth and proliferation of the beneficial bacteria, which are generally known as prebiotic approach. Thus, a combination of probiotic (live beneficial bacteria) and prebiotics (nutrients) supplementations is recommended for further investigation.

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